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(54) Title: METHODS FOR TREATMENT OF TUMORS AND TUMOR CELLS USING *EX VIVO* ACTIVATED T CELLS

(57) Abstract

Novels methods for the modulation of an immune response using treatment with T cells which are treated *ex vivo* are provided. The invention provides a solution to the problems of treating various diseases, especially those diseases that are of a malignant nature. The invention also provides methods of treating diseases on a non–malignant nature, by modulating a mammal's immune response.

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DESCRIPTION

METHODS FOR TREATMENT OF TUMORS AND TUMOR CELLS USING *EX VIVO* ACTIVATED T CELLS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of immunotherapy and oncology. More particularly, it concerns methods of inducing activated T cells *ex vivo* and methods of treating tumors and tumor cells with activated T cells in order to stimulate an immune response.

2. Description of Related Art

CD4+ T helper (T_H) cells play a critical role in regulating immune responses and may be involved in regulating immune responses against tumor cells. T_H cells can be divided into two major subclasses, T_H1 and T_H2, based on their pattern of cytokine expression and the immune responses they mediate. T_H1 cells produce interleukin-2 (IL-2), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and lymphotoxin (LT), but not IL-4 or IL-5. T_H2 cells produce IL-4 and IL-5, but not IL-2, IFN-γ, TNF-α or LT. A third class of T_H cells, T_H0, have an unrestricted pattern of cytokine expression, producing both $T_H1$and T_H2 cytokines. In experimental animal models of infection T_H1 cells regulate the cellular arm of the immune response, activating macrophages and monocytes and delayed type hypersensitivity, while T_H2 cells activate the humoral arm of the immune system, providing B cell help and stimulating IgG and IgE production. T_H0 cells can function to activate both arms of the immune response.

IFN-α, IFN-γ, IL-4 and IL-12 are capable of differentially regulating T_H cell subsets. IFN-α, IFN-γ, and IL-12 induce the differentiation of naive T_H cells into $T_H1$cells, while IL-4 is the only cytokine known to promote the differentiation of

naive cells into T_H2 cells. Furthermore, the responsiveness of differentiated T_H cells to these cytokines also varies. For instance, IFN-γ inhibits the proliferation of T_H2 but not $T_H1$cells, while T_H1, but not T_H2 cells retain IL-12 responsiveness.

While there are data to support the role of both T_H1 and T_H2 cells in anti-tumor responses, the majority of pre-clinical animal studies suggest that T_H1 responses play a key role. The ability of mice to reject the P815 tumor cell line has been shown to be largely due to the presence of CD4+ cells producing $T_H1$cytokines (Fallarino *et al.*, 1996). In these studies, mice who failed to generate a high IFN-γ T_H1 response failed to reject tumors, while those that did generate such a response rejected their tumors more efficiently. IL-12 was required for the generation of the T_H1 response. Further evidence for the role of T_H1 responses in anti-tumor immunity come from studies in mice with established experimental sarcomas (Zitvogel *et al.*, 1995). Established tumors were injected with fibroblasts engineered to secrete IL-12 or with control fibroblasts. The IL-12 secreting fibroblasts efficiently elicited a CD4 T_H1 anti-tumor response that resulted in successful control of the tumor.

Aggressive non-Hodgkin's lymphoma (NHL) is often curable with intensive combination chemotherapy, with 40-50% of patients being alive and free of disease at 5 years (Armitage, 1993; Salles *et al.*, 1994). However, patients who relapse or those with primary refractory disease have a poor prognosis. The use of dose-intensive therapy with autologous marrow or peripheral blood progenitor cell (PBPC) support has become the standard therapeutic approach for these patients. However, even with this aggressive therapy, only 30-40% of patients with relapsed/refractory NHL are alive and free of disease at 5 years (Gribben *et al.*, 1989; Philip *et al.*, 1995).

Patients with relapsed/refractory NHL with incomplete or short duration responses to dose-intensive therapy have an extremely poor prognosis with overall 5-year survival rates of 5% or less. Studies have also shown that those patients with molecular evidence of residual NHL following dose-intensive therapy and those with evidence of bone marrow or stem cell involvement with NHL at the time of reinfusion

are at a high risk of failing dose-intensive regimens (Sharp *et al.*, 1992; Gribben *et al.* 1994). Thus, patients who fail to demonstrate chemo-sensitivity to their induction regimen, or those with molecular evidence of residual disease are unlikely to achieve long-term disease-free survival with dose-intensive regimens. These "high risk" patients are thus candidates for additional complimentary therapeutic modalities along with dose-intensive therapy to improve their long term event-free survival.

SUMMARY OF THE INVENTION

The present invention relates generally to methods for the modulation of an immune response involving the treatment of a mammal with a T cell, or population of T cells. In some presently preferred embodiments, the invention relates generally to methods for the induction of an immune response involving the treatment of a mammal with a T cell, or population of T cells. The invention provides a solution to the previously-discussed problems of treating various diseases, especially those diseases that are of a malignant nature. The methods taught herein allow one to induce an immune response directed against a malignant disease.

As used herein, the phrase "induction of an immune response" denotes any measurable increase in a indicator of immune responses. Induction of an immune response includes both initiation of an immune response in a mammal that is not, at the time treatment begins, mounting an immune response. Likewise, increasing or potentiating an already ongoing immune response is induction of an immune response. The invention also provides methods of treating diseases of a non-malignant nature, by modulating a mammal's immune response. "Modulation of an immune response" is defined as any measurable change in an indicator of immune responses, in response to T cell administration.

In many cases, the mammal will have a disease, such as a malignancy, the treatment of which is expected to benefit from the induction of an immune response.

In certain cases, the mammal is a human. The present methods are anticipated to be of broad application to immunoresponsive and systemic cancers.

The present invention provides methods of treating a mammal with an immunoresponsive cancer, comprising obtaining a population of peripheral blood mononuclear cells from the mammal, activating the population of peripheral blood mononuclear cells outside of the mammal, or *ex vivo*, to obtain a population of T cells that have been activated outside of the mammal, or *ex vivo*, and administering a therapeutically effective amount of the population of T cells to the mammal, thereby inducing an immune response and treating a mammal with an immunoresponsive cancer. The "therapeutically effective amounts" for use in the invention are amounts of activated T cells effective to specifically slow progression of a tumor, to induce necrosis in at least a portion of a tumor and/or to induce tumor regression or remission upon administration to selected mammals or patients.

Immunoresponsive cancers contemplated for treatment using the methods of the present invention include, but are not limited to, lymphomas, such as non-Hodgkin's lymphoma, multiple myeloma, renal cancer, ovarian cancer, prostate cancer, low-grade lymphoma, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), sarcomas, such as osteosarcomas, lung cancer, opportunistic malignancies, such as Kaposi's sarcoma and other virally related cancers such as cervical cancer and rectal cancer, or melanoma.

In a most general sense, any cancer that responds to immunologic intervention, wherein the manipulation of the immune system yields a response to the disease, is contemplated to respond to the treatments provided herein. A number of different techniques for determination of the immunologic responsiveness of a particular cancer are available and understood by those of skill in the art. For example, leukemias and lymphomas have been shown to be immunologically responsive through the use of allogenic transplantation, melanoma has been determined to be immunologically

responsive to LAK and TIL cell technology, as well as interferons, renal cancer has been shown to be immunologically responsive to interferons and IL-2, ovarian cancer has been shown to be immunologically responsive to IL-2, and prostate cancer has been determined to be immunologically responsive using the CTLA-4 mouse model.

In particular aspects of the invention, the activation of the population of peripheral blood mononuclear cells comprises contacting the population of peripheral blood mononuclear cells with at least a first antibody and at least a second antibody. In certain embodiments, the at least a first antibody and the at least a second antibody are linked to a particle, such as a bead. Beads contemplated for use can be fabricated from a variety of different materials, for example a plastic bead such as polystyrene, or a magnetic bead. In a preferred embodiment, the antibodies are immobilized on structures (*i.e.*, beads), such that discreet points of contact are made between multiple beads and each individual T cell that is the target of costimulation. The antibodies thus immobilized costimulate individual T cells from multiple contact points. In particular aspects, the optimal number of contact points may be greater than or equal to 3. However, in certain aspects of the invention, other forms of activation, such as the use of superantigens, or the use of a protein kinase C activator, such as a phorbol ester, in combination with a calcium ionophore, such as ionomycin, are contemplated for use.

In any of the foregoing methods, the terms "antibody, naked antibody and unconjugated antibody", as used herein, refer broadly to any immunologic binding agent, such as polyclonal and monoclonal IgG, IgM, IgA, IgD and IgE antibodies. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

Polyclonal antibodies, obtained from antisera, may be employed in the invention. However, the use of monoclonal antibodies (MAbs) will generally be preferred. MAbs are recognized to have certain advantages, *e.g.*, reproducibility and

large-scale production, that makes them suitable for clinical treatment. The invention thus provides monoclonal antibodies of the murine, human, monkey, rat, hamster, rabbit and even frog or chicken origin.

As will be understood by those in the art, the immunologic binding reagents encompassed by the term "antibody" extend to all naked and unconjugated antibodies from all species, and antigen binding fragments thereof, including dimeric, trimeric and multimeric antibodies; bispecific antibodies; chimeric antibodies; human and humanized antibodies; recombinant and engineered antibodies, and fragments thereof.

The term "antibody" is thus used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art.

In certain embodiments, the antibodies employed will be "humanized" or human antibodies. "Humanized" antibodies are generally chimeric monoclonal antibodies from mouse, rat, or other non-human species, bearing human constant and/or variable region domains ("part-human chimeric antibodies"). Mostly, humanized monoclonal antibodies for use in the present invention will be chimeric antibodies wherein at least a first antigen binding region, or complementarity determining region (CDR), of a mouse, rat or other non-human monoclonal antibody is operatively attached to, or "grafted" onto, a human antibody constant region or "framework".

"Humanized" monoclonal antibodies for use herein may also be monoclonal antibodies from non-human species wherein one or more selected amino acids have been exchanged for amino acids more commonly observed in human antibodies. This can be readily achieved through the use of routine recombinant technology, particularly site-specific mutagenesis.

In some aspects of the invention, the at least a first antibody and the at least a second antibody are distinct antibodies. In certain preferred aspects, the at least a first antibody is an anti-CD3 antibody, such as the OKT3 or G19-4 antibody. In other embodiments, the at least a second antibody is an anti-CD28 antibody, such as the 9.3, KOLT-2, 15E8, 248.23.2 or EX5.3D10 antibody. In particularly preferred aspects of the present invention, the at least a first antibody is an anti-CD3 antibody and the at least a second antibody is an anti-CD28 antibody. In further aspects, an anti-CD2 antibody can be utilized in combination with an anti-CD28 antibody, an anti-CD3 antibody can be utilized in combination with IL-2 or IL-15, or an anti-B7-1 (CD80) or anti-B7-2 (CD86) antibody can be used in conjunction with an anti-CD3 antibody.

In particular embodiments of the invention, the population of T cells comprises CD4+ T cells. In certain embodiments, the population of T cells comprises predominantly CD4+ T cells, while in other embodiments, the population of T cells are only CD4+ T cells. However, in further embodiments, the population of T cells comprises CD4+ T cells and CD8+ T cells. In certain aspects of the invention, the population of T cells are not activated upon administration to the mammal. Thus, in some presently preferred embodiments, the T cell is in a quiescent or non-activated state upon administration to the mammal.

The CD4+ and/or the CD8+ T cells can be expanded or derived from a population of CD4+ and CD8+ T cells that have been expanded by treatment with antibodies. In many cases, the expansion by treatment with antibodies has occurred *ex vivo*. In preferred embodiments, the expansion by treatment with antibodies involves costimulation with two distinct populations of antibodies. Preferably one of the distinct populations of antibodies is a population of anti-CD3 antibodies, for example OKT3. Preferably, one of the distinct populations of antibodies is a population of anti-CD28 antibodies. In presently preferred embodiments, the expansion involves costimulation with a population of anti-CD3 antibodies and a

population of anti-CD28 antibodies, *via* methods described elsewhere in this application.

In particular aspects of the present invention, the immune response comprises the production of T cells in the mammal. In other aspects, the immune response comprises the production of CD8+ T cells in the mammal. In preferred embodiments, the population of T cells administered to the mammal are predominantly or totally CD4+ T cells, and the immune response comprises the production of CD8+ T cells in the mammal. And in other preferred embodiments, the population of T cells are predominantly CD4+ T cells, and the immune response comprises the production of CD8+ T cells directed against the immunoresponsive cancer in the mammal.

In addition to malignant diseases, CD4+ T cells may be used to treat patients with other disorders of the immune system, for example, those patients with hyper-immune states or immunodeficiencies. In these cases, the invention involves modulating the immune response that is mediated by CD4+ cells, in some cases by turning off or turning down an immune response or by reorganizing the immune response. For example, CD4+ T cells may be employed to treat diseases such as diabetes, lupus, or rheumatoid arthritis. Likewise, T cells may be employed to prevent organ rejection after transplantation. In cases of implant rejection prevention, there will often be benefits derived from T cell treatment prior to the transplantation procedure.

In some cases, the T cells will be administered in combination with other treatment modalities, for example, chemotherapy, antibiotic therapy, antibody therapy, radiation therapy, transplants, *etc.* In some preferred methods of practicing the invention, the patient may be given a stem cell transplant in conjunction with the T cell treatment. In the treatment of malignancies, administration of CD4+ T cells may serve to correct inappropriate activation of the immune system. In other diseases, the there are also problems associated with defective immune responses. Therefore, there may be advantages to immunosuppressing the patient prior to the

T cell therapy. By immunosuppressing the patient, the inappropriate immune response depressed, and the T cell therapy can then affect appropriate activation of the immune system. In cases where a stem cell transplant is given, the chemotherapy associated with the transplant procedure immunosuppresses the patient prior to the CD4+ T cell administration.

Thus, in further embodiments of the invention, the methods further comprise immunosuppression of the mammal prior to administration of the population of the T cells to the mammal. Any of a number of techniques for immunosuppression are contemplated for use in the present methods and are known to those of skill in the art, including, but not limited to, administration of chemotherapy, radiation therapy, azathioprine, cyclophosphamide, rapamycin, corticosteroids, such as prednisone, cyclosporine A, FK506, purine analogs and related inhibitors, such as fluderilzene, 2-chloro-deoxyadenosine, mercaptopurine (6-mercaptopurine), thioguanine (6-thioguanine) and pentostatin (2-deoxycoformycin), alkylating agents, for example nitrogen mustards such as mechlorethamine (HN_2), cyclophosphamide, ifosfamide, melphalan (L-sarcolysin) and chlorambucil, ethylenimenes and methylmelamines such as hexamethylmelamine and thiotepa, alkyl sulfonates such as busulfan, nitrosoureas such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU) and streptozocin (streptozotocin) and triazines such as dacarbazine (DTIC; dimethyltriazenoimidazolecarboxamide), or anti-T cell monoclonal antibodies to the mammal.

In yet other aspects of the invention, the methods further comprise administering a stem cell transplant to the mammal. In preferred embodiments, the stem cells comprise CD34+ cells. The invention also encompasses a combination therapy comprising the co-administration of CD4+ T cells with cytokines or other biological compounds that can modulate or specify the phenotype of the CD4+ T cells or the CD8+ T cells. For example treatment with IL-2, IL-12, IL-4, IL-10, or IL-6 can be used to modify function of cell product produced.

The present invention also provides methods of treating a mammal with non-Hodgkin's lymphoma, comprising obtaining a population of peripheral blood mononuclear cells from the mammal, activating the population of peripheral blood mononuclear cells outside of the mammal to obtain a population of T cells that have been activated outside of the mammal, and administering the population of T cells to the mammal, thereby inducing an immune response and treating a mammal with non-Hodgkin's lymphoma.

In preferred embodiments of the invention, the activation of the population of peripheral blood mononuclear cells comprises contacting the population of peripheral blood mononuclear cells with at least a first anti-CD3 antibody and at least a first anti-CD28 antibody. In particularly preferred aspects, the at least a first anti-CD3 antibody and the at least a first anti-CD28 antibody are linked to a magnetic bead. In other preferred embodiments, the population of T cells comprises predominantly CD4+ T cells.

The present invention further provides methods of treating a mammal with T cells that have a defective cytokine profile, comprising obtaining a population of peripheral blood mononuclear cells from the mammal, activating the population of peripheral blood mononuclear cells outside of the mammal to obtain a population of T cells that have been activated outside of the mammal, and administering the population of T cells to the mammal, thereby treating a mammal with T cells that have a defective cytokine profile. In preferred embodiments, the activation of the population of peripheral blood mononuclear cells comprises costimulation with a population of anti-CD3 antibodies and a population of anti-CD28 antibodies.

Further provided are methods of inducing lymphocytosis in a mammal, comprising obtaining a population of peripheral blood mononuclear cells from the mammal, activating the population of peripheral blood mononuclear cells outside of the mammal to obtain a population of T cells that have been activated outside of the

mammal, and administering the population of T cells to the mammal, thereby inducing lymphocytosis in the mammal.

Following long-standing patent law convention, the words "a" and "an," as used in this specification, including the claims, denotes "one or more." Specifically, the use of "comprising," "having," or other open language in claims that claim a combination or method employing "an object," denotes that "one or more of the object" may be employed in the claimed method or combination.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and FIG. 1B. CD3+28 T cell expansion from PBMCs. A typical pre-clinical expansion of CD3+28+ T cells is shown. **FIG. 1A** shows an expansion in X-VIVO 15® with 5% autologous human serum, and **FIG. 1B** is an expansion in the same medium with 100 U/ml interleukin-2 (Cetus) added. In both cases it can be noted that the method supports the logarithmic growth and expansion of CD3+ T cells, and that CD4+ T cells kinetically expand greater than do CD8+ T cells.

FIG. 2. Clinical CD3+28 T cell expansion (Patient SE 8558-06). A clinical expansion of CD3+28 T cells is shown. Similar results are obtained to the preclinical scale expansions such as that shown in FIG. 1A and FIG. 1B. Specifically, CD4+ T cells are expanded preferentially over CD8+ T cells.

FIG. 3. Immunophenotypic analysis of CD3+28 costimulated T cell expansion culture. The time points S1D0, S1D4, S1D9 and S1D13 correspond to days 1, 5, 10 and 14 of culture, respectively. The starting population of cells shown here is typical for these patients. In general, 40-60% of the cells are monocytes

(CD14+), 10-40% are T cells (CD3+), 5-15% are B cells (CD19+), 5-30% are CD4+ T cells (CD3+CD4+) and 1-15% are CD8+ T cells (CD3+CD8+). By day 5 (S1D4) nearly all of the cells in the culture are T cells, with a few monocytes persisting. After this, all of the cells are either CD4+ or CD8+ T cells. By day 14, >99% of all of the cells in the culture are CD3+ T cells and 90% are CD4+. B cells are not expanded and are rapidly lost from the cultures.

FIG. 4. Recovery of lymphoid and myeloid cell compartments of patient (AS 8558-03) following dose-intensive chemotherapy with CD34-detected PBPC support and CD3+28 costimulated T cells. Blood cell counts were measured during the recovery phase from dose-intensive therapy and CD34-selected PBPC support and CD3+28 costimulated T cell reinfusion. The costimulated T cells were reinfused on day 13 on the x-axis. Note that neutrophils (POLY + BAND COUNT) recovered by day 15 and have remained relatively constant since that time. In contrast, the lymphocytes had an initial phase of recovery beginning at day 20 and then a steep second phase beginning at day 30. This second phase is an absolute and relative lymphocytosis, which is currently still sustained. Notably, these cells are atypical, predominantly CD8+ with an activated phenotype and show monoclonal or oligoclonal Vβ repertoire usage (FIG. 10A, FIG. 10B, FIG. 10C, FIG. 10D, FIG. 11A, FIG. 11B, FIG. 11C and FIG. 11D). Thus, this second phase correlates with a specific immune response that is likely initiated and maintained by the reinfused CD3+28 costimulated T cells.

FIG. 5. Differential of Patient (AS 8558-03) following dose-intensive chemotherapy with CD34-detected PBPC support and CD3+28 costimulated T cells. Shown is the differential of patient AS 8558-03's blood counts over the same period as FIG. 4. Note the relative lymphocytosis with the first (days 21-27) and second (days 30-72) waves of lymphocytes recovery. Another interesting feature found in all 4 evaluable patients is that eosinophilia (days 21-30) precedes the second wave of lymphocytosis. This is useful to predict when this second wave of lymphocytes will appear.

FIG. 6. Recovery of lymphoid and myeloid cell compartments of patient (CR 8558-05) following dose-intensive chemotherapy with CDE34-detected PBPC support and CD3+28 costimulated T cells. Blood cell counts were measured during the recovery phase from dose-intensive therapy and CD34-selected PBPC support and CD3+28 costimulated T cell reinfusion. The costimulated T cells were reinfused on day 13 on the x-axis. Note that neutrophils (POLY + BAND COUNT) recovered by day 14 and after peaking at day 35 have leveled off at approximately 1800/μl. In contrast, the lymphocytes had an initial phase of recovery beginning at day 20 and then a second phase beginning at day 29. In contrast to patient AS 8558-03 (FIG. 4), this patient's second phase has a relative, although not absolute lymphocytosis. Like AS 8558-03, this patient's relative lymphocytosis is currently still sustained. Notably, as with AS 8558-03, this patent's lymphocytes are atypical, predominantly CD8+ with an activated phenotype and show monoclonal or oligoclonal Vβ repertoire usage.

FIG. 7. Differential of Patient (CR 8558-05) following dose-intensive chemotherapy with CDE34-detected PBPC support and CD3+28 costimulated T cells. Shown is the differential of patient AS 8558-05's blood counts over the same period as FIG. 6. Note the relative lymphocytosis with the first (days 20-29) and second (days 35-57) waves of lymphocytes recovery. Again, as noted in FIG. 5, eosinophilia (days 21-30) predicts the second wave of lymphocytosis.

FIG. 8. Recovery of lymphoid and myeloid cell compartments of patient (SE/8558-06) following dose-intensive chemotherapy with CD34-selected PBPC support and CD3+28 costimulated T cells. Blood cell counts were measured during the recovery phase from dose-intensive therapy with CD34-selected PBPC support and CD3+28 costimulated T cell reinfusion. The costimulated T cells were reinfused on day 13. The neutrophils (POLY + BAND COUNT) recovered by day 14 and, after peaking at day 32, leveled off at approximately 4000/ml. In contrast to the previous

patients AS/8558-03 and CR/8558-05 (FIG. 4, FIG. 6), the lymphocytes remained relatively constant.

FIG. 9. Differential of Patient SE/8558-06 following dose-intensive chemotherapy with CD34-selected PBPC support and CD3+28 costimulated T cells. Shown is the differential of patient SE/8558-06's blood counts over the same period as FIG. 8. Note that although the total lymphocytes appear relatively constant as plotted in FIG. 5, there is a gradual increase in the % lymphocytes in the differential (days 48-64) which, as noted in FIG. 5 and FIG. 7 is preceded by eosinophilia (days 22-32).

FIG. 10A, FIG. 10B, FIG. 10C and FIG. 10D. Analysis of Vβ20 usage in CD3+28 costimulated T cells after 14 days of culture (day of reinfusion) and in peripheral blood T cells 30 days after reinfusion. Vβ T cell antigen receptor usage was determined by PCR. FIG. 10A and FIG. 10B show the Vβ20 usage for the CD3+28 costimulated T cells after 14 days of stimulation (at the time of reinfusion into patient AS 8558-03). The analysis shows the expected gaussian distribution for a polyclonal population of T cells. FIG. 10C and FIG. 10D show the Vβ20 usage for patient AS 8558-03's peripheral blood T cells that were collected 30 days after CD3+28 costimulated T cell reinfusion. In contrast to FIG. 10A and FIG. 10B, FIG. 10C and FIG. 10D show a single peak representing a monoclonal population of T cells.

FIG. 11A, FIG. 11B, FIG. 11C and FIG. 11D. Analysis of Vβ18 usage in CD3+28 costimulated T cells after 14 days of culture (day of reinfusion) and in peripheral blood T cells 30 days after reinfusion. Vβ T cell antigen receptor usage was determined by PCR. FIG. 11A and FIG. 11B show the Vβ18 usage for the CD3+28 costimulated T cells after 14 days of stimulation (at the time of reinfusion into patient AS 8558-03). The analysis shows the expected gaussian distribution for a polyclonal population of T cells. FIG. 11C and FIG. 11D show the Vβ18 usage for patient AS 8558-03's peripheral blood T cells that were collected 30 days after

CD3+28 costimulated T cell reinfusion. In contrast to FIG. 11A and FIG. 11B, FIG. 11C and FIG. 11D show two peaks representing an oligoclonal population of T cells.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

In designing a novel biologic therapy for immunoresponsive cancers, the inventors have developed and refined methods for the *ex vivo* expansion of activated CD4+ and CD8+ T cells. Immunoresponsive cancers are defined herein as any cancer that responds to immunologic intervention, wherein the manipulation of the immune system yields a response to the disease. In general, cancers are shown to be immunoresponsive using any of a number of different techniques. For example, certain leukemias and lymphomas have been shown to be immunologically responsive through the use of allogenic transplantation, melanoma has been demonstrated to be immunologically responsive to LAK and TIL cell technology, as well as interferons, renal cancer has been determined to be immunologically responsive to interferons and IL-2, ovarian cancer has been shown to be immunologically responsive to IL-2, and prostate cancer has been determined to be immunologically responsive using the CTLA-4 mouse model (see, *e.g.*, Cancer principles and practice of oncology. Lippicott-Raven, Philadelphia, 1997, incorporated herein by reference).

An exemplary use of this therapy is as an adjunct therapy in patients undergoing dose-intensive therapy for relapsed non-Hodgkin's lymphoma (NHL). The anti-tumor activity of these expanded activated cells is measured, and presented herein. The T helper subset profile was examined in patients at the time they initially present with NHL. *In vitro* cell culture studies to identify cytokines which modulate T helper responses in these patients was also performed.

In certain aspects of the invention, a novel system is used to drive the *ex vivo* expansion of CD4+ and CD8+ T cells. In this system, antibodies to the T cell accessory molecules CD3 and CD28, immobilized to a magnetic bead, were used to provide a costimulatory signal to drive the logarithmic growth and expansion of

CD3+CD28+ human T cells. Previous T cell culturing systems (*i.e.*, LAK or TIL cell technology) used in human immunotherapy trials have predominantly resulted in the growth and expansion of CD8+ effector T cells. This CD3+28 costimulation system is the first available method to allow the large scale expansion and activation of polyclonal human CD4+ T cells *ex vivo*, along with CD8+ T cells. Specifically, a clinical trial was conducted delivering escalating doses of these *ex vivo* expanded CD3+28 costimulated T cells to patients undergoing dose-intensive therapy with autologous CD34-selected peripheral blood progenitor cell (PBPC) support for relapsed NHL (BB IND 6914). The ability to eliminate tumors and tumor cells was demonstrated, as measured by both conventional and newly developed assays specific to this protocol. However, other methods of activation of T cells are also contemplated for use in certain aspects of the present invention (June *et al.*, PCT Patent Applications WO 94/29436 and WO 95/33823, each of which is specifically incorporated herein by reference).

One approach to improving the results of therapy for patients with relapsed/refractory NHL is to combine another therapeutic modality such as biologic therapy or immunomodulation with dose-intensive therapy. This may result in the eradication of minimal residual disease that has failed to respond completely to the dose-intensive regimen. Studies have shown that those patients with molecular evidence of residual NHL following dose-intensive therapy and those with evidence of bone marrow or stem cell involvement with NHL at the time of reinfusion are at a high risk of failing dose-intensive regimens (Zwicky *et al.*, 1996). Thus, molecular evidence of residual disease appears to be useful in identifying patients who may not achieve long-term disease-free survival with dose-intensive regimens alone and are thus candidates for additional complimentary therapeutic modalities along with dose-intensive therapy.

A. **Antibodies**

1. **Polyclonal Antibodies**

Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference). To prepare polyclonal antisera an animal is immunized with an immunogenic composition, and antisera collected from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, mouse, rat, hamster, guinea pig or goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen; subcutaneous, intramuscular, intradermal, intravenous, intraperitoneal and intrasplenic. The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired titer level is obtained, the immunized animal can be bled and the serum isolated and stored. The animal can also be used to generate monoclonal antibodies.

As is well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary adjuvants include complete Freund's adjuvant, a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*; incomplete Freund's adjuvant; and aluminum hydroxide adjuvant.

It may also be desired to boost the host immune system, as may be achieved by associating the immunogen to a carrier. Exemplary carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as

ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers.

2. Monoclonal Antibodies

Various methods for generating monoclonal antibodies (MAbs) are also now very well known in the art. The most standard monoclonal antibody generation techniques generally begin along the same lines as those for preparing polyclonal antibodies (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference). A polyclonal antibody response is initiated by immunizing an animal with an immunogenic composition and, when a desired titer level is obtained, the immunized animal can be used to generate MAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in US. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with the selected immunogen composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep and frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61; incorporated herein by reference), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen

lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984; each incorporated herein by reference). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F, 4B210 or one of the above listed mouse cell lines; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6, are all useful in connection with human cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 4:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976; each incorporated herein by reference), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977; incorporated herein by reference). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986; incorporated herein by reference).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated

indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

MAbs produced by either means will generally be further purified, *e.g.*, using filtration, centrifugation and various chromatographic methods, such as HPLC or affinity chromatography, all of which purification techniques are well known to those of skill in the art. These purification techniques each involve fractionation to separate the desired antibody from other components of a mixture. Analytical methods particularly suited to the preparation of antibodies include, for example, protein A-Sepharose and/or protein G-Sepharose chromatography.

3. Antibodies from Phagemid Libraries

Recombinant technology now allows the preparation of antibodies having the desired specificity from recombinant genes encoding a range of antibodies (Van Dijk *et al.*, 1989; incorporated herein by reference). Certain recombinant techniques involve the isolation of the antibody genes by immunological screening of combinatorial immunoglobulin phage expression libraries prepared from RNA isolated from the spleen of an immunized animal (Morrison *et al.*, 1986; Winter and Milstein, 1991; each incorporated herein by reference).

For such methods, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells. The advantages of this approach over

conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination, which further increases the percentage of appropriate antibodies generated.

One method for the generation of a large repertoire of diverse antibody molecules in bacteria utilizes the bacteriophage lambda as the vector (Huse *et al.*, 1989; incorporated herein by reference). Production of antibodies using the lambda vector involves the cloning of heavy and light chain populations of DNA sequences into separate starting vectors. The vectors are subsequently combined randomly to form a single vector that directs the co-expression of heavy and light chains to form antibody fragments. The heavy and light chain DNA sequences are obtained by amplification, preferably by PCR™ or a related amplification technique, of mRNA isolated from spleen cells (or hybridomas thereof) from an animal that has been immunized with a selected antigen. The heavy and light chain sequences are typically amplified using primers that incorporate restriction sites into the ends of the amplified DNA segment to facilitate cloning of the heavy and light chain segments into the starting vectors.

Another method for the generation and screening of large libraries of wholly or partially synthetic antibody combining sites, or paratopes, utilizes display vectors derived from filamentous phage such as M13, fl or fd. These filamentous phage display vectors, referred to as "phagemids", yield large libraries of monoclonal antibodies having diverse and novel immunospecificities. The technology uses a filamentous phage coat protein membrane anchor domain as a means for linking gene-product and gene during the assembly stage of filamentous phage replication, and has been used for the cloning and expression of antibodies from combinatorial libraries (Kang *et al.*, 1991; Barbas *et al.*, 1991; each incorporated herein by reference).

This general technique for filamentous phage display is described in U.S. Patent 5,658,727, incorporated herein by reference. In a most general sense, the

method provides a system for the simultaneous cloning and screening of pre-selected ligand-binding specificities from antibody gene repertoires using a single vector system. Screening of isolated members of the library for a pre-selected ligand-binding capacity allows the correlation of the binding capacity of an expressed antibody molecule with a convenient means to isolate the gene that encodes the member from the library.

Linkage of expression and screening is accomplished by the combination of targeting of a fusion polypeptide into the periplasm of a bacterial cell to allow assembly of a functional antibody, and the targeting of a fusion polypeptide onto the coat of a filamentous phage particle during phage assembly to allow for convenient screening of the library member of interest. Periplasmic targeting is provided by the presence of a secretion signal domain in a fusion polypeptide. Targeting to a phage particle is provided by the presence of a filamentous phage coat protein membrane anchor domain (*i.e.*, a cpIII- or cpVIII-derived membrane anchor domain) in a fusion polypeptide.

The diversity of a filamentous phage-based combinatorial antibody library can be increased by shuffling of the heavy and light chain genes, by altering one or more of the complementarity determining regions of the cloned heavy chain genes of the library, or by introducing random mutations into the library by error-prone polymerase chain reactions. Additional methods for screening phagemid libraries are described in U.S. Patent No. 5,580,717; 5,427,908; 5,403,484; and 5,223,409, each incorporated herein by reference.

Another method for the screening of large combinatorial antibody libraries has been developed, utilizing expression of populations of diverse heavy and light chain sequences on the surface of a filamentous bacteriophage, such as M13, fl or fd (U.S. Patent No. 5,698,426; incorporated herein by reference). Two populations of diverse heavy (Hc) and light (Lc) chain sequences are synthesized by polymerase chain reaction (PCR™). These populations are cloned into separate M13-based vector

containing elements necessary for expression. The heavy chain vector contains a gene VIII (gVIII) coat protein sequence so that translation of the heavy chain sequences produces gVIII-Hc fusion proteins. The populations of two vectors are randomly combined such that only the vector portions containing the Hc and Lc sequences are joined into a single circular vector.

The combined vector directs the co-expression of both Hc and Lc sequences for assembly of the two polypeptides and surface expression on M13 (U.S. Patent No. 5,698,426; incorporated herein by reference). The combining step randomly brings together different Hc and Lc encoding sequences within two diverse populations into a single vector. The vector sequences donated from each independent vector are necessary for production of viable phage. Also, since the pseudo gVIII sequences are contained in only one of the two starting vectors, co-expression of functional antibody fragments as Lc associated gVIII-Hc fusion proteins cannot be accomplished on the phage surface until the vector sequences are linked in the single vector.

Surface expression of the antibody library is performed in an amber suppressor strain. An amber stop codon between the Hc sequence and the gVIII sequence unlinks the two components in a non-suppressor strain. Isolating the phage produced from the non-suppressor strain and infecting a suppressor strain will link the Hc sequences to the gVIII sequence during expression. Culturing the suppressor strain after infection allows the coexpression on the surface of M13 of all antibody species within the library as gVIII fusion proteins (gVIII-Fab fusion proteins). Alternatively, the DNA can be isolated from the non-suppressor strain and then introduced into a suppressor strain to accomplish the same effect.

The surface expression library is screened for specific Fab fragments that bind preselected molecules by standard affinity isolation procedures. Such methods include, for example, panning (Parmley and Smith, 1988; incorporated herein by reference), affinity chromatography and solid phase blotting procedures. Panning is preferred, because high titers of phage can be screened easily, quickly and in small

volumes. Furthermore, this procedure can select minor Fab fragments species within the population, which otherwise would have been undetectable, and amplified to substantially homogenous populations. The selected Fab fragments can be characterized by sequencing the nucleic acids encoding the polypeptides after amplification of the phage population.

Another method for producing diverse libraries of antibodies and screening for desirable binding specificities is described in U.S. Patent No. 5,667,988 and U.S. Patent No. 5,759,817, each incorporated herein by reference. The method involves the preparation of libraries of heterodimeric immunoglobulin molecules in the form of phagemid libraries using degenerate oligonucleotides and primer extension reactions to incorporate the degeneracies into the CDR regions of the immunoglobulin variable heavy and light chain variable domains, and display of the mutagenized polypeptides on the surface of the phagemid. Thereafter, the display protein is screened for the ability to bind to a preselected antigen.

The method for producing a heterodimeric immunoglobulin molecule generally involves (1) introducing a heavy or light chain V region-coding gene of interest into the phagemid display vector; (2) introducing a randomized binding site into the phagemid display protein vector by primer extension with an oligonucleotide containing regions of homology to a CDR of the antibody V region gene and containing regions of degeneracy for producing randomized coding sequences to form a large population of display vectors each capable of expressing different putative binding sites displayed on a phagemid surface display protein; (3) expressing the display protein and binding site on the surface of a filamentous phage particle; and (4) isolating (screening) the surface-expressed phage particle using affinity techniques such as panning of phage particles against a preselected antigen, thereby isolating one or more species of phagemid containing a display protein containing a binding site that binds a preselected antigen.

A further variation of this method for producing diverse libraries of antibodies and screening for desirable binding specificities is described in U.S. Patent No. 5,702,892, incorporated herein by reference. In this method, only heavy chain sequences are employed, the heavy chain sequences are randomized at all nucleotide positions which encode either the CDRI or CDRIII hypervariable region, and the genetic variability in the CDRs is generated independent of any biological process.

In the method, two libraries are engineered to genetically shuffle oligonucleotide motifs within the framework of the heavy chain gene structure. Through random mutation of either CDRI or CDRIII, the hypervariable regions of the heavy chain gene were reconstructed to result in a collection of highly diverse sequences. The heavy chain proteins encoded by the collection of mutated gene sequences possessed the potential to have all of the binding characteristics of an immunoglobulin while requiring only one of the two immunoglobulin chains.

Specifically, the method is practiced in the absence of the immunoglobulin light chain protein. A library of phage displaying modified heavy chain proteins is incubated with an immobilized ligand to select clones encoding recombinant proteins that specifically bind the immobilized ligand. The bound phage are then dissociated from the immobilized ligand and amplified by growth in bacterial host cells. Individual viral plaques, each expressing a different recombinant protein, are expanded, and individual clones can then be assayed for binding activity.

4. Antibodies from Human Lymphocytes

In vitro immunization, or antigen stimulation, may also be used to generate a human antibody. Such techniques can be used to stimulate peripheral blood lymphocytes from normal, healthy subjects. Such "*in vitro* immunization" involves antigen-specific activation of non-immunized B lymphocytes, generally within a mixed population of lymphocytes (mixed lymphocyte cultures, MLC). *In vitro* immunizations may also be supported by B cell growth and differentiation factors and

lymphokines. The antibodies produced by these methods are often IgM antibodies (Borrebaeck *et al.*, 1986; incorporated herein by reference).

Another method has been described (U.S. Patent No. 5,681,729, incorporated herein by reference) wherein human lymphocytes that mainly produce IgG (or IgA) antibodies can be obtained. The method involves, in a general sense, transplanting human lymphocytes to an immunodeficient animal so that the human lymphocytes "take" in the animal body; immunizing the animal with a desired antigen, so as to generate human lymphocytes producing an antibody specific to the antigen; and recovering the human lymphocytes producing the antibody from the animal. The human lymphocytes thus produced can be used to produce a monoclonal antibody by immortalizing the human lymphocytes producing the antibody, cloning the obtained immortalized human-originated lymphocytes producing the antibody, and recovering a monoclonal antibody specific to the desired antigen from the cloned immortalized human-originated lymphocytes.

The immunodeficient animals that may be employed in this technique are those that do not exhibit rejection when human lymphocytes are transplanted to the animals. Such animals may be artificially prepared by physical, chemical or biological treatments. Any immunodeficient animal may be employed. The human lymphocytes may be obtained from human peripheral blood, spleen, lymph nodes, tonsils or the like.

The "taking" of the transplanted human lymphocytes in the animals can be attained by merely administering the human lymphocytes to the animals. The administration route is not restricted and may be, for example, subcutaneous, intravenous or intraperitoneal. The dose of the human lymphocytes is not restricted, and can usually be 10^6 to 10^8 lymphocytes per animal. The immunodeficient animal is then immunized with the desired antigen.

After the immunization, human lymphocytes are recovered from the blood, spleen, lymph nodes or other lymphatic tissues by any conventional method. For example, mononuclear cells can be separated by the Ficoll-Hypaque (specific gravity: 1.077) centrifugation method, and the monocytes removed by the plastic dish adsorption method. The contaminating cells originating from the immunodeficient animal may be removed by using an antiserum specific to the animal cells. The antiserum may be obtained by, for example, immunizing a second, distinct animal with the spleen cells of the immunodeficient animal, and recovering serum from the distinct immunized animal. The treatment with the antiserum may be carried out at any stage. The human lymphocytes may also be recovered by an immunological method employing a human immunoglobulin expressed on the cell surface as a marker.

By these methods, human lymphocytes mainly producing IgG and IgA antibodies specific to one or more selected antigen(s) can be obtained. Monoclonal antibodies are then obtained from the human lymphocytes by immortalization, selection, cell growth and antibody production.

5. Transgenic Mice Containing Human Antibody Libraries

Recombinant technology is now available for the preparation of antibodies. In addition to the combinatorial immunoglobulin phage expression libraries disclosed above, another molecular cloning approach is to prepare antibodies from transgenic mice containing human antibody libraries. Such techniques are described in U.S. Patent No. 5,545,807, incorporated herein by reference.

In a most general sense, these methods involve the production of a transgenic animal that has inserted into its germline genetic material that encodes for at least part of an immunoglobulin of human origin or that can rearrange to encode a repertoire of immunoglobulins. The inserted genetic material may be produced from a human source, or may be produced synthetically. The material may code for at least part of a

known immunoglobulin or may be modified to code for at least part of an altered immunoglobulin.

The inserted genetic material is expressed in the transgenic animal, resulting in production of an immunoglobulin derived at least in part from the inserted human immunoglobulin genetic material. It is found the genetic material is rearranged in the transgenic animal, so that a repertoire of immunoglobulins with part or parts derived from inserted genetic material may be produced, even if the inserted genetic material is incorporated in the germline in the wrong position or with the wrong geometry.

The inserted genetic material may be in the form of DNA cloned into prokaryotic vectors such as plasmids and/or cosmids. Larger DNA fragments are inserted using yeast artificial chromosome vectors (Burke *et al.*, 1987; incorporated herein by reference), or by introduction of chromosome fragments (Richer and Lo, 1989; incorporated herein by reference). The inserted genetic material may be introduced to the host in conventional manner, for example by injection or other procedures into fertilized eggs or embryonic stem cells.

In preferred aspects, a host animal that initially does not carry genetic material encoding immunoglobulin constant regions is utilized, so that the resulting transgenic animal will use only the inserted human genetic material when producing immunoglobulins. This can be achieved either by using a naturally occurring mutant host lacking the relevant genetic material, or by artificially making mutants *e.g.*, in cell lines ultimately to create a host from which the relevant genetic material has been removed.

Where the host animal carries genetic material encoding immunoglobulin constant regions, the transgenic animal will carry the naturally occurring genetic material and the inserted genetic material and will produce immunoglobulins derived from the naturally occurring genetic material, the inserted genetic material, and mixtures of both types of genetic material. In this case the desired immunoglobulin

can be obtained by screening hybridomas derived from the transgenic animal, *e.g.*, by exploiting the phenomenon of allelic exclusion of antibody gene expression or differential chromosome loss.

Once a suitable transgenic animal has been prepared, the animal is simply immunized with the desired immunogen. Depending on the nature of the inserted material, the animal may produce a chimeric immunoglobulin, *e.g.* of mixed mouse/human origin, where the genetic material of foreign origin encodes only part of the immunoglobulin; or the animal may produce an entirely foreign immunoglobulin, *e.g.* of wholly human origin, where the genetic material of foreign origin encodes an entire immunoglobulin.

Polyclonal antisera may be produced from the transgenic animal following immunization. Immunoglobulin-producing cells may be removed from the animal to produce the immunoglobulin of interest. Preferably, monoclonal antibodies are produced from the transgenic animal, *e.g.*, by fusing spleen cells from the animal with myeloma cells and screening the resulting hybridomas to select those producing the desired antibody. Suitable techniques for such processes are described herein.

In an alternative approach, the genetic material may be incorporated in the animal in such a way that the desired antibody is produced in body fluids such as serum or external secretions of the animal, such as milk, colostrum or saliva. For example, by inserting *in vitro* genetic material encoding for at least part of a human immunoglobulin into a gene of a mammal coding for a milk protein and then introducing the gene to a fertilized egg of the mammal, *e.g.*, by injection, the egg may develop into an adult female mammal producing milk containing immunoglobulin derived at least in part from the inserted human immunoglobulin genetic material. The desired antibody can then be harvested from the milk. Suitable techniques for carrying out such processes are known to those skilled in the art.

The foregoing transgenic animals are usually employed to produce human antibodies of a single isotype, more specifically an isotype that is essential for B cell maturation, such as IgM and possibly IgD. Another preferred method for producing human antibodies is described in U.S. Patent No. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and 5,770,429; each incorporated by reference, wherein transgenic animals are described that are capable of switching from an isotype needed for B cell development to other isotypes.

In the development of a B lymphocyte, the cell initially produces IgM with a binding specificity determined by the productively rearranged V_H and V_L regions. Subsequently, each B cell and its progeny cells synthesize antibodies with the same L and H chain V regions, but they may switch the isotype of the H chain. The use of mu or delta constant regions is largely determined by alternate splicing, permitting IgM and IgD to be coexpressed in a single cell. The other heavy chain isotypes (gamma, alpha, and epsilon) are only expressed natively after a gene rearrangement event deletes the C mu and C delta exons. This gene rearrangement process, termed isotype switching, typically occurs by recombination between so called switch segments located immediately upstream of each heavy chain gene (except delta). The individual switch segments are between 2 and 10 kb in length, and consist primarily of short repeated sequences.

For these reasons, it is preferable that transgenes incorporate transcriptional regulatory sequences within about 1-2 kb upstream of each switch region that is to be utilized for isotype switching. These transcriptional regulatory sequences preferably include a promoter and an enhancer element, and more preferably include the 5' flanking (*i.e.*, upstream) region that is naturally associated (*i.e.*, occurs in germline configuration) with a switch region. Although a 5' flanking sequence from one switch region can be operably linked to a different switch region for transgene construction, in some embodiments it is preferred that each switch region incorporated in the transgene construct have the 5' flanking region that occurs immediately upstream in the naturally occurring germline configuration. Sequence information relating to

immunoglobulin switch region sequences is known (Mills *et al.*, 1991; Sideras *et al.*, 1989; each incorporated herein by reference).

In the method described in U.S. Patent No. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and 5,770,429, the human immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B-cell development, leading to isotype switching. Accordingly, in this method, these transgenes are constructed so as to produce isotype switching and one or more of the following: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

An important requirement for transgene function is the generation of a primary antibody repertoire that is diverse enough to trigger a secondary immune response for a wide range of antigens. The rearranged heavy chain gene consists of a signal peptide exon, a variable region exon and a tandem array of multi-domain constant region regions, each of which is encoded by several exons. Each of the constant region genes encode the constant portion of a different class of immunoglobulins. During B-cell development, V region proximal constant regions are deleted leading to the expression of new heavy chain classes. For each heavy chain class, alternative patterns of RNA splicing give rise to both transmembrane and secreted immunoglobulins.

The human heavy chain locus consists of approximately 200 V gene segments spanning 2 Mb, approximately 30 D gene segments spanning about 40 kb, six J segments clustered within a 3 kb span, and nine constant region gene segments spread out over approximately 300 kb. The entire locus spans approximately 2.5 Mb of the distal portion of the long arm of chromosome 14. Heavy chain transgene fragments containing members of all six of the known V_H families, the D and J gene segments, as well as the mu, delta, gamma 3, gamma 1 and alpha 1 constant regions are known

(Berman *et al.*, 1988; incorporated herein by reference). Genomic fragments containing all of the necessary gene segments and regulatory sequences from a human light chain locus is similarly constructed.

The expression of successfully rearranged immunoglobulin heavy and light transgenes usually has a dominant effect by suppressing the rearrangement of the endogenous immunoglobulin genes in the transgenic nonhuman animal. However, in certain embodiments, it is desirable to effect complete inactivation of the endogenous Ig loci so that hybrid immunoglobulin chains comprising a human variable region and a non-human (*e.g.*, murine) constant region cannot be formed, for example by trans-switching between the transgene and endogenous Ig sequences. Using embryonic stem cell technology and homologous recombination, the endogenous immunoglobulin repertoire can be readily eliminated. In addition, suppression of endogenous Ig genes may be accomplished using a variety of techniques, such as antisense technology.

In other aspects of the invention, it may be desirable to produce a trans-switched immunoglobulin. Antibodies comprising such chimeric trans-switched immunoglobulins can be used for a variety of applications where it is desirable to have a non-human (*e.g.*, murine) constant region, *e.g.*, for retention of effector functions in the host. The presence of a murine constant region can afford advantages over a human constant region, for example, to provide murine effector functions (*e.g.*, ADCC, murine complement fixation) so that such a chimeric antibody may be tested in a mouse disease model. Subsequent to the animal testing, the human variable region encoding sequence may be isolated, *e.g.*, by PCR amplification or cDNA cloning from the source (hybridoma clone), and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic use.

6. Humanized Antibodies

Human antibodies generally have at least three potential advantages for use in human therapy. First, because the effector portion is human, it may interact better with the other parts of the human immune system, *e.g.*, to destroy target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). Second, the human immune system should not recognize the antibody as foreign. Third, the half-life in the human circulation will be similar to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

Various methods for preparing human antibodies are provided herein. In addition to human antibodies, "humanized" antibodies have many advantages. "Humanized" antibodies are generally chimeric or mutant monoclonal antibodies from mouse, rat, hamster, rabbit or other species, bearing human constant and/or variable region domains or specific changes. Techniques for generating a so-called "humanized" antibody are well known to those of skill in the art.

Humanized antibodies also share the foregoing advantages. First, the effector portion is still human. Second, the human immune system should not recognize the framework or constant region as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody. Third, injected humanized antibodies, as opposed to injected mouse antibodies, will presumably have a half-life more similar to naturally occurring human antibodies, also allowing smaller and less frequent doses.

A number of methods have been described to produce humanized antibodies. Controlled rearrangement of antibody domains joined through protein disulfide bonds to form new, artificial protein molecules or "chimeric" antibodies can be utilized (Bobrzecka *et al.*, 1980; Konieczny *et al.*, 1981; each incorporated herein by reference). Recombinant DNA technology can also be used to construct gene fusions between DNA sequences encoding mouse antibody variable light and heavy chain

domains and human antibody light and heavy chain constant domains (Morrison *et al.*, 1984; incorporated herein by reference).

DNA sequences encoding the antigen binding portions or complementarity determining regions (CDR's) of murine monoclonal antibodies can be grafted by molecular means into the DNA sequences encoding the frameworks of human antibody heavy and light chains (Jones *et al.*, 1986; Riechmann *et al.*, 1988; each incorporated herein by reference). The expressed recombinant products are called "reshaped" or humanized antibodies, and comprise the framework of a human antibody light or heavy chain and the antigen recognition portions, CDR's, of a murine monoclonal antibody.

Another method for producing humanized antibodies is described in U.S. Patent No. 5,639,641, incorporated herein by reference. The method provides, via resurfacing, humanized rodent antibodies that have improved therapeutic efficacy due to the presentation of a human surface in the variable region. In the method: (1) position alignments of a pool of antibody heavy and light chain variable regions is generated to give a set of heavy and light chain variable region framework surface exposed positions, wherein the alignment positions for all variable regions are at least about 98% identical; (2) a set of heavy and light chain variable region framework surface exposed amino acid residues is defined for a rodent antibody (or fragment thereof); (3) a set of heavy and light chain variable region framework surface exposed amino acid residues that is most closely identical to the set of rodent surface exposed amino acid residues is identified; (4) the set of heavy and light chain variable region framework surface exposed amino acid residues defined in step (2) is substituted with the set of heavy and light chain variable region framework surface exposed amino acid residues identified in step (3), except for those amino acid residues that are within 5Å of any atom of any residue of the complementarity determining regions of the rodent antibody; and (5) the humanized rodent antibody having binding specificity is produced.

A similar method for the production of humanized antibodies is described in U.S. Patent No. 5,693,762; 5,693,761; 5,585,089; and 5,530,101, each incorporated herein by reference. These methods involve producing humanized immunoglobulins having one or more complementarity determining regions (CDR's) and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. Each humanized immunoglobulin chain usually comprises, in addition to the CDR's, amino acids from the donor immunoglobulin framework that are capable of interacting with the CDR's to effect binding affinity, such as one or more amino acids that are immediately adjacent to a CDR in the donor immunoglobulin or those within about 3Å as predicted by molecular modeling. The heavy and light chains may each be designed by using any one, any combination, or all of the various position criteria described in U.S. Patent No. 5,693,762; 5,693,761; 5,585,089; and 5,530,101, each incorporated herein by reference. When combined into an intact antibody, the humanized immunoglobulins are substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the original antigen.

An additional method for producing humanized antibodies is described in U.S. Patent 5,565,332 and 5,733,743, each incorporated herein by reference. This method combines the concept of humanizing antibodies with the phagemid libraries also described in detail herein. In a general sense, the method utilizes sequences from the antigen binding site of an antibody or population of antibodies directed against an antigen of interest. Thus for a single rodent antibody, sequences comprising part of the antigen binding site of the antibody may be combined with diverse repertoires of sequences of human antibodies that can, in combination, create a complete antigen binding site.

The antigen binding sites created by this process differ from those created by CDR grafting, in that only the portion of sequence of the original rodent antibody is likely to make contacts with antigen in a similar manner. The selected human sequences are likely to differ in sequence and make alternative contacts with the

antigen from those of the original binding site. However, the constraints imposed by binding of the portion of original sequence to antigen and the shapes of the antigen and its antigen binding sites, are likely to drive the new contacts of the human sequences to the same region or epitope of the antigen. This process has therefore been termed "epitope imprinted selection" (EIS).

Starting with an animal antibody, one process results in the selection of antibodies that are partly human antibodies. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or after alteration of a few key residues. Sequence differences between the rodent component of the selected antibody with human sequences could be minimized by replacing those residues that differ with the residues of human sequences, for example, by site directed mutagenesis of individual residues, or by CDR grafting of entire loops. However, antibodies with entirely human sequences can also be created. EIS therefore offers a method for making partly human or entirely human antibodies that bind to the same epitope as animal or partly human antibodies respectively. In EIS, repertoires of antibody fragments can be displayed on the surface of filamentous phase and the genes encoding fragments with antigen binding activities selected by binding of the phage to antigen.

Additional methods for humanizing antibodies contemplated for use in the present invention are described in U.S. Patent No. 5,750,078; 5,502,167; 5,705,154; 5,770,403; 5,698,417; 5,693,493; 5,558,864; 4,935,496; and 4,816,567, each incorporated herein by reference.

7. Antibody Fragments

Irrespective of the source of the original antibody, either the intact antibody, antibody multimers, or any one of a variety of functional, antigen-binding regions of the antibody may be used in the present invention. Exemplary functional regions include scFv, Fv, Fab', Fab and $F(ab')_2$ fragments of the antibodies. Techniques for

preparing such constructs are well known to those in the art and are further exemplified herein.

The choice of antibody construct may be influenced by various factors. For example, prolonged half-life can result from the active readsorption of intact antibodies within the kidney, a property of the Fc piece of immunoglobulin. IgG based antibodies, therefore, are expected to exhibit slower blood clearance than their Fab' counterparts. However, Fab' fragment-based compositions will generally exhibit better tissue penetrating capability.

Fab fragments can be obtained by proteolysis of the whole immunoglobulin by the non-specific thiol protease, papain. Papain must first be activated by reducing the sulphydryl group in the active site with cysteine, 2-mercaptoethanol or dithiothreitol. Heavy metals in the stock enzyme should be removed by chelation with EDTA (2 mM) to ensure maximum enzyme activity. Enzyme and substrate are normally mixed together in the ratio of 1:100 by weight. After incubation, the reaction can be stopped by irreversible alkylation of the thiol group with iodoacetamide or simply by dialysis. The completeness of the digestion should be monitored by SDS-PAGE and the various fractions separated by protein A-Sepharose or ion exchange chromatography.

The usual procedure for preparation of $F(ab')_2$ fragments from IgG of rabbit and human origin is limited proteolysis by the enzyme pepsin. The conditions, 100x antibody excess w/w in acetate buffer at pH 4.5, 37°C, suggest that antibody is cleaved at the C-terminal side of the inter-heavy-chain disulfide bond. Rates of digestion of mouse IgG may vary with subclass and it may be difficult to obtain high yields of active $F(ab')_2$ fragments without some undigested or completely degraded IgG. In particular, IgG_{2b} is highly susceptible to complete degradation. The other subclasses require different incubation conditions to produce optimal results, all of which is known in the art.

Digestion of rat IgG by pepsin requires conditions including dialysis in 0.1 M acetate buffer, pH 4.5, and then incubation for four hours with 1% w/w pepsin; IgG_1 and IgG_{2a} digestion is improved if first dialyzed against 0.1 M formate buffer, pH 2.8, at 4°C, for 16 hours followed by acetate buffer. IgG_{2b} gives more consistent results with incubation in staphylococcal V8 protease (3% w/w) in 0.1 M sodium phosphate buffer, pH 7.8, for four hours at 37°C.

8. Bispecific Antibodies

In certain methods of the present invention, bispecific antibodies are preferred for use. The preparation of bispecific antibodies is also well known in the art. One preparative method involves the separate preparation of antibodies having specificity for a first particular component, on the one hand, and a second particular component on the other. Peptic $F(ab'\gamma)_2$ fragments from the two chosen antibodies are then generated, followed by reduction of each to provide separate $Fab'\gamma_{SH}$ fragments. The SH groups on one of the two partners to be coupled are then alkylated with a cross-linking reagent, such as o-phenylenedimaleimide, to provide free maleimide groups on one partner. This partner may then be conjugated to the other by means of a thioether linkage, to give the desired $F(ab'\gamma)_2$ heteroconjugate (Glennie *et al.*, 1987; incorporated herein by reference). Other approaches, such as cross-linking with SPDP or protein A may also be carried out.

Another method for producing bispecific antibodies is by the fusion of two hybridomas to form a quadroma. As used herein, the term "quadroma" is used to describe the productive fusion of two B cell hybridomas. Using now standard techniques, two antibody producing hybridomas are fused to give daughter cells, and those cells that have maintained the expression of both sets of clonotype immunoglobulin genes are then selected.

A preferred method of generating a quadroma involves the selection of an enzyme deficient mutant of at least one of the parental hybridomas. This first mutant hybridoma cell line is then fused to cells of a second hybridoma that had been lethally

exposed, *e.g.*, to iodoacetamide, precluding its continued survival. Cell fusion allows for the rescue of the first hybridoma by acquiring the gene for its enzyme deficiency from the lethally treated hybridoma, and the rescue of the second hybridoma through fusion to the first hybridoma. Preferred, but not required, is the fusion of immunoglobulins of the same isotype, but of a different subclass. A mixed subclass antibody permits the use if an alternative assay for the isolation of a preferred quadroma.

Microtiter identification embodiments, FACS, immunofluorescence staining, idiotype specific antibodies, antigen binding competition assays, and other methods common in the art of antibody characterization may be used to identify preferred quadromas. Following the isolation of the quadroma, the bispecific antibodies are purified away from other cell products. This may be accomplished by a variety of antibody isolation procedures, known to those skilled in the art of immunoglobulin purification (see, *e.g.*, Antibodies: A Laboratory Manual, 1988; incorporated herein by reference). Protein A or protein G sepharose columns are preferred.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

CD3+28 Costimulation for the *ex vivo* Expansion and Activation of CD4+ T Cells

The inventors developed a novel system to drive the expansion of CD4+ T cells (Levine *et al.*, 1996; Carroll *et al.*, 1997). This system uses antibodies to the T

cell accessory molecules CD3 and CD28 to drive polyclonal logarithmic growth of human T cells *in vitro*. The inventors also have developed a clinical protocol to use *ex vivo* expanded autologous human CD4+ T cells as an immunotherapeutic adjunct to dose-intensive therapy for patients with high risk relapsed/refractory NHL (described below). In this protocol, patients with relapsed/refractory NHL who are high risk candidates for dose-intensive therapy, defined as chemo-insensitive disease in relapse or primary refractory disease, undergo a steady-state leukapheresis to collect PBMCs as a source of T cells, and then undergo PBPC mobilization with chemotherapy plus cytokines. After collection of a minimum number of CD34+ progenitor cells, the product of the harvest undergoes a CD34 positive selection step. The selected CD34+ cells are used for hematologic rescue after the patient has received a dose-intensive chemotherapy regimen. The positive selection step depletes the autologous T cells in the PBPC thus delaying recovery of the endogenous T cell compartment following the autologous stem cell transplant. Peripheral blood mononuclear cells (PBMCs) from the cryopreserved steady-state pheresis product are cultured for 14 days in the CD3+28 costimulation system that promotes the preferential expansion of CD4+ T cells.

Pre-clinical data indicated that 2.5-3 $\log_{10}$ expansion of T cells was achieved in 14 days of culture in this system (FIG. 1A, FIG. 1B, FIG. 2 and FIG. 3). The T cell expansion system used in this application drives the polyclonal expansion of CD3+CD28+T cells. Although both CD4- and CD8-positive T cells are expanded, the CD4+ cells are expanded to a greater extent than the CD8+ cells (FIG. 1A, FIG. 1B, FIG. 2 and FIG. 3). The cytokine-secretion phenotype of the expanded CD4+ cells generally has a T_H1 pattern. The doses of T cells were escalated in patients using a phase I study design (described in detail below).

EXAMPLE 2

Immunological profile of patients receiving CD3+28 costimulated T cells

Eight patients were treated with CD3+28 costimulated T cells following dose-intensive chemotherapy and PBPC reinfusion on Protocol 8558 [BB IND 6914]. Toxicity was mild with no grade 2 or higher early (24 hr.) toxicities (see protocol) associated with CD3+28 T cell reinfusion of up to 2×10^9 total cells. The immunologic consequences of reinfused T cells were greater than expected. The cell manufacturing and release data are summarized in Table 1 below. The adverse events are summarized in Table 2, below.

TABLE 1

Cell Manufacturing and Release Data

Patient #	Target Cell Dose	Actual Cell Dose	Gram Stain	Culture	Myco-plasma	Endotoxin	Residual Beads	Cell Viability
RB/8558-02	2×10^9	1.04×10^9	NEG	NEG	NEG	NEG	1000	81%
AS/8558-03	2×10^9	3.90×10^8	NEG	NEG	NEG	NEG	4550	80%
CR/8558-05	2×10^9	1.38×10^8	NEG	NEG	NEG	NEG	333	80%
SE/8558-06	2×10^9	1.35×10^9	NEG	NEG	NEG	NEG	5863	88%
LB/8558-07	2×10^9	-	ND	POS. Gram stain: mod Gram negative bacilli. Culture ID: Many Stenotrophomonas maltophilia	NEG	POS (>1 EU/ml)	ND	ND
JP/8558-09	5×10^9	2.5×10^9	NEG	NEG	NEG	NEG	2499	85%
AO/8558-10	5×10^9	-	NEG	NEG	NEG	NEG	17356	ND
FM/8558-11	5×10^9	-	NEG	POS	NEG	NEG	2560	85%
DF/8558-12	5×10^9	2.4×10^9	NEG		NEG	NEG	328	88%
SV/8558-13	5×10^9	5×10^9	NEG	NEG	NEG	NEG	155,000	82%
RL/8558-14	5×10^9	5×10^9	NEG	NEG	NEG	NEG	165,000	91%
AG/8558-15	5×10^9	5.2×10^8	NEG	NEG	NEG	NEG	14,785	85%

TABLE 2

Summary of Adverse Events

Patient #	Age/ Sex	Dose (Level)	Adverse Events: CD3+28 cell-related	Adverse Events: Not CD3+28 cell-related	Adverse Events: Other
AS/8558-03	60/M	3.90×10^8 (1)	Nausea: Grade 1; Fevers: Grade 2; Fatigue: Grade 1; Anorexia: Grade 1	Infection: Grade 3; Nausea: Grade 3; Vomiting: Grade 2; Diarrhea: Grade 1; Stomatitis: Grade 1; Alopecia: Grade: 2; Neuro: Grade 2; Fatigue: Grade 1; Anorexia: Grade 1; Hepatic: Grade 1; Renal: Grade 1	Pulmonary: Grade 3 (Pre-transplant DLCO)
CR/8558-05	52/F	1.38×10^8 (1)	Fatigue: Grade 1; Fevers: Grade 1	Pain: Grade 2 (abd); Nausea: Grade 2; Vomiting: Grade 1; Diarrhea: Grade 2; Stomatitis: Grade 2; Alopecia: Grade 2; Neuro: Grade 2; Skin: Grade 2; Edema: Grade 1; Fevers: Grade 2; Fatigue: Grade 1; Anorexia: Grade 1; Hepatic: Grade 4; Renal: Grade 1	
SE/8558-06	33/M	1.35×10^9 (1)	None	Nausea: Grade 1; Vomiting: Grade 1; Diarrhea: Grade 1; Stomatitis: Grade 1; Alopecia: Grade 2; Neuro: Grade 2 (Syncopal episode); Edema: Grade 1; Fevers: Grade 1; Anorexia: Grade 1	None

TABLE 2 - Continued

JP/8558-09	54/M	2.5×10^9 (2)	Fevers: Grade 2 Fatigue: Grade 1	Nausea: Grade 2; Vomiting: Grade 1; Diarrhea: Grade 1; Stomatitis: Grade 1; Alopecia: Grade 2; Fatigue: Grade 1; Anorexia: Grade 1; Hepatic: Grade 3	Neuro: Grade 3 Fatigue: Grade 1
DF/8558-12	33/F	2.4×10^9 (2)		Infection: Grade 2; Pulmonary: Grade 5 (poss. cont. by CD3+28 T cells); Hepatic: Grade 5 (poss. cont. by CD3+28 T cells)	
SV/8558-13	53/M	2.4×10^9 (2)	Fevers, Fatigue, Skin: All Grade 2;	Infection: Grade 2; Neuro: Grade 1 (possible contribution by CD3 + 28 T cells)	
RL/8558-14	42/M	5×10^9 (2)	Flu-like Sxs: Grade 2	Infection: Grade 3; Anemia: Grade 2; Thrombocytopenia: Grade 3	Cardiac, Pain (Ab.): Grade 3; (poss. cont. by CD3+28 T cells); Anorexia: Grade 2
AG/8558-15	70/M	5×10^9 (2)		Infection: Grade 1	

In the eight patients who were evaluable, the following observations were made. First, certain patients demonstrated an absolute or relative lymphocytosis within 2-4 weeks of CD3+28 T cell reinfusion (FIG. 4, FIG. 5, FIG. 6 and FIG. 7). Further, the phenotype of the lymphocytes that predominate in these patients surprisingly was not the same as the phenotype of the CD3+28 costimulated T cells that were reinfused into each of these patients. While the reinfused T cells were predominantly (60 to 90%) CD4+ (FIG. 1A, FIG. 1B, FIG. 2 and FIG. 3), the lymphocytes predominate in these patients 2-4 weeks post CD3+28 T cell reinfusion were predominantly (85-95%) CD8+.

The cytokine expression profile of the CD4+ and CD8+ T cells that were present during the period of lymphocytosis was determined in the 4 patients evaluated. For the profile of CD4+ T cells, T cells (CD3 perCP+ cells) were gated and then analyzed for CD4+ surface staining and intracellular cytokine expression as described herein. The results showed that 68% of the CD4+ T cells expressed TNF-α, 25% expressed IL-4 and 85% expressed IFN-γ. Isotype-matched negative control antibodies were used to set quadrants. Thus, the majority of activated cells express the T_H1 cytokines TNF-α and IFN-γ. For the profile of CD8+ T cells, T cells (CD3 perCP+ cells) were gated and then analyzed for CD8+ surface staining and intracellular cytokine expression as described herein. These results showed that 48% of the CD8+ T cells expressed TNF-α, 4.6% expressed IL-4 and 70% expressed IFN-γ. Isotype-matched negative control antibodies were used to set quadrants. These data indicate that a significant fraction of the circulating CD8+ T cells are activated to produce T_C1 cytokines. This, combined with the CD8+ clonality data (FIG. 10A, FIG, 10B, FIG. 10C, FIG. 10D, FIG. 11A, FIG. 11B, FIG. 11C and FIG. 11D) suggest that these cells represent specifically activated effector cells. The low overall number of activated CD4+ T cells compared to that of the CD8+ T cells suggest that most of the activated CD4+ T cells are not circulating, but may be sequestered in lymphoid compartments or at sites of tumor.

The significance of cytokine production profiles by human CD8+ T cells is not as well characterized, but cells with a type 1 cytokine secretion profile (T_C1) may be more active cytolytically than those with a T_C2 profile (Maggi *et al.*, 1997). T cell repertoire analysis using PCR™ for the Vβ T cell antigen receptor reveals that the Vβ usage of the CD3+28 costimulated product has a gaussian (polyclonal) distribution, indicating the relative nonspecific polyclonal nature of the expanded cell product. This is expected since costimulation through CD3+28 should globally act and expand T cells and hopefully overcome activation defects that may be present in these patients (Ochoa and Longo, 1995). In contrast, the Vβ usage of the CD8+ T cells that emerge in the wave of lymphocytosis are not gaussian and are often monoclonal or oligoclonal. Taken together, these data are within the inventors' hypothesis that the CD8+ lymphocytes which emerge in the patient 2-4 weeks following CD3+28 costimulated T cell reinfusion are antigen or tumor specific effector cells that are being driven by the CD3+28 costimulated CD4+ T cells. The inventors expect that the specificity of these CD8+ clonal T cell populations can be determined if these same cells are present in biopsies of residual tumor in select patients and then by testing the ability of these cells to kill autologous transformed B cell lines.

These data are remarkably similar to animal studies in which mice with tumors have alterations in T cell signaling pathways that prevent normal immunologic recognition and elimination of tumor cells (Mizoguchi *et al.*, 1992). In mice with tumors, there is a progressive, sequential development of functional alterations in T cells, and also a progressive loss of T_H1 cell populations (Correa *et al.*, 1997; Ghosh *et al.*, 1995). Activation of splenocytes or CD4+ T cells from these tumor-bearing mice with anti-CD3 + IL-2 overcomes these T cell activation defects, generating tumor-specific responses when the activated cells are adoptively transferred into mice following bone marrow transplantation or treatment with cyclophosphamide (Katsanis *et al.*, 1994; Saxton *et al.*, 1997).

EXAMPLE 3

Immunological Consequences and Anti-tumor Efficacy of *ex vivo* Expanded and Activated CD3+28 costimulated CD4+ and CD8+ T cells

A clinical protocol was developed to examine expanding CD4+ T cells for human immunotherapeutic use. The safety of these cells when delivered in escalating doses in the post-transplant setting, the dose tolerance of these cells in this patient population, and the efficacy of these cells in reducing the presence of molecularly detectable residual disease and relapse rates in these patients were evaluated. The clinical protocol was developed using CD3+28 costimulated T cells following dose-intensive therapy. The specific methods for the isolation and growth of these cells are provided below.

Patients were selected according to the eligibility criteria outlined herein. After undergoing leukapheresis, the PBPC were separated into CD34-positive and CD34-negative fractions. The CD34-positive fraction was used for hematopoietic rescue of patients following dose-intensive chemotherapy. The CD34-negative fraction was used to start the CD3+28 costimulation cultures as described fully below. The CD3+28 costimulation cultures were started on the same day that patients received back their CD34-positive PBPCs. Fourteen days later, the CD3+28 costimulated T cells were harvested, counted, and a specific dose was reinfused into the patient. The study design is a phase I study in which cohorts of three patients received escalating doses of autologous CD3+28 costimulated T cells.

This study assessed administration of *ex vivo* expanded CD3+28 costimulated T cells following dose-intensive therapy and CD34-selected peripheral blood progenitor cell support in patients with relapsed or refractory B cell NHL, and assessed the toxicity of escalating doses of CD3+28 costimulated T cells in this patient population. This study also assessed the immune responsiveness of patient's T cells prior to therapy, in response to *ex vivo* CD3+28 costimulation, and following adoptive therapy with costimulated T cells.

Specimens were collected from peripheral blood, bone marrow and cell products in the study to measure T_H subsets by flow cytometry using intracellular cytokine staining. Measuring pre- and post-infusion T_H subsets helped to determine the immunophysiologic consequences of delivering these *ex vivo* derived CD4+ T cells to patients with relapsed NHL. Finally, correlation of molecular responses with T cell infusion or with T_H subset polarization helped to identify useful surrogate immunologic markers for anti-tumor activity.

A. Collection of Peripheral Blood

Clinical specimens of peripheral blood were collected at the time of phlebotomy for standard clinical laboratory tests. All patients gave written informed consent.

B. Isolation of PBMCs and Separation of B and T cells *in vitro*

Mononuclear cells were isolated from peripheral whole blood by density gradient centrifugation on Ficoll-Hypaque (BioWhittaker, Inc.). The washed mononuclear cells then were separated into T cell-rich and B cell-rich fractions either by purification on a human T cell enrichment column (R&D Systems) according to the manufacturer's directions, or by E rosetting with aminoethylisothiouronium hydrobromide (AET) treated sheep red blood cells. The B cells and T cells were used as described below.

C. Intracellular Cytokine Staining and Analysis by Flow Cytometry

T cells were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co.) and the ionophore ionomycin (I; Sigma Chemical Co.), and the cytokine expression pattern (*i.e.* T_H1 or T_H2) was measured. T cells (0.5-1.0 x 10^6 cells/ml) were cultured at 37°C/5%CO_2 in RPMI-1640 (BioWhittaker, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Inc.), PMA+I (25 ng/ml and 1 mg/ml, respectively) and 2 mM monensin (Calbiochem). Control cultures were set up identically with the exception that the PMA+I was

omitted. Monensin was added to the culture to inhibit cytokine secretion, resulting in the intracellular accumulation of cytokines.

At the end of 4 hours, surface antigen-specific fluorescent-conjugated monoclonal antibodies were incubated with the cells at room temperature for 15-30 min. FACS lysing solution (Becton Dickinson) was added to lyse any remaining red cells and to partially fix the T cells. The solution was washed off the cells, and then the cells were incubated with FACS permeabilizing solution (Becton Dickinson) at room temperature for 10 minutes in order to permeabilize the lymphocyte membranes prior to intracellular immunofluorescence staining. The cells were washed, and then incubated with fluorscent-conjugated intracellular monoclonal antibodies at room temperature for 30 minutes. After a second wash, the cells were resuspended in PBS with 1% BSA and analyzed by three color flow cytometry on a FACScan (Becton Dickinson) flow cytometer. Surface antigen specific monoclonal antibodies that were used included perCP-anti-CD3 (Becton Dickinson), FITC-anti-CD4, and FITC-anti-CD8 (both from DAKO). Intracellular monoclonal antibodies that were used included PE-anti-IL-2, PE-anti-IL-4, PE-anti-IFN-γ, and PE-anti-TNF-α (all from Becton Dickinson).

EXAMPLE 4

CD34+ Selection

Dose-intensive therapy is supported by mobilized PBPC. The use of this stem cell source has been associated with a more rapid hematologic recovery (Zimmerman *et al.*, 1995). In addition, it has been suggested that patients transplanted with PBPC may have a survival advantage over those receiving harvested bone marrow (Vose *et al.*, 1993). PBPC are mobilized with cyclophosphamide and GM-CSF plus G-CSF. The use of high dose cyclophosphamide to mobilize PBPC provides therapeutic effect by using an active agent which is not duplicated in the transplant preparative regimen. In certain aspects, the combination of GM-CSF and G-CSF is more effective than either growth factor alone in mobilizing progenitor cells (Winter *et al.*, 1996).

One approach to improving the results of therapy for patients with "high risk" relapsed/refractory NHL is to combine another therapeutic modality such as immunotherapy with dose-intensive therapy. This results in the eradication of disease that has failed to respond completely to the dose-intensive regimen. In the present study, escalating doses of *ex vivo* expanded CD4+CD28+ T cells are used as an immunotherapeutic adjunct to the dose-intensive regimen. The product of CD34 positive selection is then used as a source of progenitor cells for rescue from the dose-intensive regimen and the steady-state leukapheresis product as the source of CD4+CD28+ T cells to target for *ex vivo* expansion.

The protocol expands a population of CD4+ T cells *ex vivo* from patients with relapsed/refractory non-Hodgkin's lymphoma (NHL), and delivers these expanded cells as complimentary therapy for patients whose tumors failed to respond to induction chemotherapy. This protocol is also contemplated for use in other immunotherapeutic protocols targeting patients whose immunoresponsive tumors are not responsive to chemotherapy alone.

All of the published adoptive immunotherapeutic trials for treating patients with malignancy have used T cells which are predominantly CD8+. This was due, in part, to the absence of a system available for the large scale growth and expansion of CD4+ T cells.

A. Preclinical Data on CD4+ T Cell Expansion

The proliferative potential of T cells expanded *in vitro* is a major consideration for adoptive immunotherapy. Antigen-specific and polyclonal $CD8^+$ T cells have been successfully expanded *in vitro* by the addition of IL-2 or anti-CD3 Ab + IL-2. However, mixed populations of $CD4^+$ and $CD8^+$ T cells stimulated in this manner will eventually result in a population that is all or mostly $CD8^+$. Further, the long-term growth of $CD4^+$ T cells has necessitated the addition of exogenous lymphokines and

allogeneic feeder cells, which precludes a large-scale expansion of CD4+ T cells for the treatment of disease.

The two-signal model of lymphocyte activation states that lymphocytes require the delivery of both an antigen-specific signal as well as a simultaneous co-stimulatory signal. In the absence of a co-stimulatory signal, interaction of the T cell receptor with the antigen-MHC complex may cause T cell clonal anergy or deletion. Data from many laboratories indicate that CD28 can provide an important co-stimulatory signal. The inventors developed a novel method for preferentially expanding CD4+ T cells independent of exogenous cytokines or feeder cells using anti-CD3 Ab plus anti-CD28 Ab conjugated to magnetic beads.

Autocrine growth in normal donors is maintained for a 4-6 $\log_{10}$-fold expansion. These cells remain polyclonal and predominantly $CD4^+$. In some patients, the addition of IL-2 enhances growth of lymphocytes, and this is therefore an optional step in certain aspects. Activated cells secrete predominantly cytokines associated with T helper type 1 function.

The inventors have recently used this system to preferentially expand CD4+CD28+ T cells from the CD34 negative selection product from normal peripheral allogeneic donors as well as autologous patient-donors with malignancy. The CD34 negative selection product is used to seed the initial cultures without further selection or manipulation. Cultures set up in this fashion show a 2-3 $\log_{10}$ expansion over 14 days with a final cell culture product that is 85-90% CD4+ and 10-15% CD8+ T cells. Thus, the targeted numbers of CD4+ T cells for reinfusion are expanded from 1×10^8 to 1×10^9 CD34 negative selection product cells without any further processing.

B. Clinical Data on the Safety of *ex vivo* Expanded CD4+ T Cells

Thus far two HIV infected patients have received a total of three infusions of *ex vivo* expanded CD4+ T cells. The first patient received 2×10^9 expanded CD4+

cells on one occasion followed by 1.1×10^{10} cells on a second reinfusion. The second patient received 3×10^{10} cells. Toxicity has been mild with night sweats (without fever) and a headache being the only reported side effects 12 hours after the infusion of the 1.1×10^{10} cell dose in patient 1, and fever and red-man syndrome in patient 2 at the 3×10^{10} cell dose.

C. Study Design

This is a single arm open label feasibility study. High risk patients (defined below) undergo standard dose-intensive chemotherapy for relapsed/refractory NHL (consisting of BCNU, VP-16, Ara-C and cyclophosphamide (BEAC)), with CD34 selected PBPC support. Fourteen days following reinfusion of CD34 selected PBPCs, the patients undergo reinfusion of *ex vivo* expanded CD4+ T cells. The dose of T cells ranges from 2×10^9 to 5×10^{10} cells.

1. Eligibility Criteria

Relapsed NHL is defined as patients with recurrent disease after CR (defined below), but not more than 5 years after achieving CR, unless tumor can be shown to be identical to the original clone. Refractory refers to primary refractory disease.

"High risk" disease is defined as less than a PR (*i.e.*, SD or PD) to a standard pre-transplant induction regimen, creatinine < 1.5 or calculated creatinine clearance of > 60 ml/min, total bilirubin, AST and ALT < 1.5× upper limit of normal (unless due to Gilbert's for bilirubin).

2. Pretreatment Evaluation

Pathology material is reviewed to confirm diagnosis. Paraffin blocks or frozen material from initial biopsy and relapse should be provided within 30 days of entry. A small portion is used to develop tumor-specific oligonucleotide probes to the CDR3 region of the immunoglobulin heavy chain gene. A CT scan of chest/abdomen/pelvis and SPECT-gallium scan are performed, along with a bone marrow aspirate and biopsy. Five to ten cubic centimeters (cc) of bone marrow aspirate is obtained at the

time of disease staging to measure tumor cells on the bone marrow mononuclear cell fraction and on 21-day bone marrow mononuclear cell cultures using a PCR-based assay with tumor specific probes. Twenty cc of heparinized peripheral blood is collected at the time of pre-transplant evaluation, and tumor cell contamination is determined on the peripheral blood mononuclear cell fraction using a PCR-based assay with tumor specific probes.

3. PBPC Mobilization and Leukapheresis (PBPC Collections)

Stem cells are mobilized into the peripheral blood using a standard regimen. Apheresis is performed with the Fenwal CS3000 Plus cell collector, or equivalent, using a large bore double lumen catheter as venous access with total processing of approximately 10-12 liters per session. The small volume collection chamber (SVCC) of the CS3000 Plus is used for the collection.

Mobilization is assessed by monitoring levels of circulating CD34+ cells in peripheral blood, and is considered successful when circulating CD34+ cells $\geq$ 5/μl. Following successful mobilization, and when WBC$\geq$ 1000/μl, patients undergo leukapheresis daily. Endpoints for successful PBPC harvest include harvest of at least 5×10^6 CD34+cells/kg. From this, 1.0×10^6 CD34+ cells/kg are cryopreserved without further manipulation, and stored for rescue of the patient in case of graft failure. Failure to meet the backup collection after 6 days of leukapheresis or the total collection minima in 12 or fewer days of apheresis disqualifies the patient for the CD34 positive selection procedure and T cell expansion. The standard procedure for operation of the CS3000 and collection of cells is provided below.

The backup aliquot of mononuclear cell suspensions obtained by apheresis is cryopreserved without further manipulation. The peripheral stem cells are stored according to the Stem Cell Cryolab standard. Details on the cryopreservation procedure are outlined below.

4. CD34+ Selection from PBSC Apheresis Collection

Selection is done using the Isolex 300 System for positive selection of CD34 cells. The system is comprised of the following components: a monoclonal antibody directed against the CD34 antigen expressed on hematopoietic progenitor/stem cells (this antigen is selectively expressed by a small percentage of bone marrow and a peripheral blood mononuclear cells); the Isolex™ 300i Magnetic Cell Separator; paramagnetic microspheres coated with sheep anti-murine antibody; and a non-enzymatic stem cell releasing agent to separate the targeted CD34+ cells from the paramagnetic microspheres. The Isolex™ 300i Magnetic Cell Separator is a device which controls all of the steps in the separation process, including washing and sensitization of the target cells, separation of the paramagnetic microspheres/target cells from the mononuclear cell suspensions, and release of the target cells form the paramagnetic microspheres.

Components of the Magnetic Cell Separator include: a primary magnet whose adjustable position is controlled automatically; a stationary secondary magnet; a series of clamps and pumps; a support module for the washing chamber; and a graphic LCD display with a touch screen used as the operator interface. The associated Isolex™ 300i Disposable Set comprises a sterile biocompatible fluid path for the cells. The main components of the set include the washing chamber, the mixing/separation chamber (which interfaces with the primary magnet), and the secondary chamber, which interfaces with the secondary magnet. The sheep anti-murine antibody coated microspheres (Dynabeads M-450 sheep anti-mouse) bind murine immunoglobulin and provide the mechanism for targeting the antibody coated cells for selection. The non-enzymatic stem cell releasing agent displaces the cells from the bead-cell rosettes, allowing the target cells to be collected and the beads to be retained by the magnet.

CD34+ cells are isolated from the PBPC collections of the test group using the Baxter Isolex 300 System. All procedures are performed using aseptic technique. The selection procedure includes specific steps for PBPC preparation, sensitization of the mononuclear cells with the 9069 anti-CD34 monoclonal antibody, rosetting of target

cells and paramagnetic beads, release of target cells from the beads, and washing of isolated CD34+ cells. Details of the selection procedure are outlined below.

For the PBPC preparation, a platelet wash is performed on the apheresis product prior to the isolation procedure. This occurs using the spinning membrane assembly, which is part of the disposable set (wash chamber). Sensitization of the mononuclear cells is performed with 2.5 mg of anti-CD34 antibody in a total volume of 100 mL. Sensitization is performed for fifteen minutes at room temperature, after which the cells are washed to remove excess/unbound antibody. Freshly prepared Dynal® paramagnetic microspheres (SAM IgG) are then added to washed, sensitized cells. After incubation at room temperature, the bead-cell complexes are separated from the unbound cells using the primary magnet. The bead/target cell complexes are retained in the separation chamber and the non-target cells are washed away. The non-targeted fraction is collected using aseptic technique for T cell expansion. Using the stem cell releasing agent, the CD34+ cells are displaced, releasing them from the beads. This requires incubation at room temperature, after which the target cells are collected using the primary magnet to hold the beads in the chamber. The final CD34+ product is assayed for sterility with bacterial cultures and sensitivity, mycoplasma and fungal cultures.

D. *Ex Vivo* Expansion of CD4+CD28+ T Lymphocytes

1. Cell Culture of PBMCs

A steady-state pheresis product obtained prior to stem cell mobilization is used as the source of PBMCs that are cultured in a system which facilitates the preferential expansion of CD4+CD28+ T lymphocytes. Cells are seeded into a 300 ml Baxter Lifecell Flask, or Baxter 2417 flask if the starting amount of cells is at least 100×10^6 cells. Details on the use of Baxter Lifecell® Flask and Solution Transfer Pump to culture cells are provided below.

The cells are grown in X-VIVO 15® supplemented with 5% autologous serum collected and prepared during apheresis. Magnetic beads (BB IND 6675) containing

immobilized anti-CD3 (OKT3) and anti-CD28 (9.3) antibodies are washed and added at a 3:1 Bead: CD3+ cell ratio. The cultures are maintained for 14 days prior to harvesting for preparation for reinfusion. The cells are counted daily and fresh medium is added to maintain the cells at a density of about 0.75-2 x 10^6 per ml as outlined below.

The phenotype of the *ex vivo* expanded cells is determined at the time of initial seeding, at 7 days of expansion, and then on day 14 of expansion at the time of harvesting. The cells are stained with anti-CD3, anti-CD4 and anti-CD8, and analyzed by flow cytometry to determine the total number and relative frequency of total T cells (CD3+), as well as CD4+ and CD8+ T cells.

2. Preparation of Cultured Cells for Infusion

The cells are expanded *ex vivo* for 14 days and then processed for reinfusion on day 14. The magnetic beads are removed using a Baxter Fenwal Maxsep® magnetic cell separation system. It is preferable to efficiently remove the microbeads prior to reinfusion into the patient to prevent or reduce the possibility of undesirable side effects that may be caused by the infusion of the antibody-coated microbeads. Details of the bead removal process are provided below.

Cell culture harvest is performed the day of scheduled cell reinfusion and once the cultures have undergone the magnetic cell separation process for removal of the microbeads used for cell stimulation. The cells are harvested, washed and resuspended in Plasmalyte A containing 1% human serum albumin with the Baxter Fenwal cell harvester, as described below.

E. Reinfusion of Expanded T Cells

The total dose of reinfused T cells is based on the number of CD3+ cells as determined by the total cell count and flow cytometry with anti-CD3. After fourteen days of expansion this constitutes ≥99% of the total cells in the culture.

The starting CD3+ cell dose is 2×10^9 cells in 100-250 ml Plasmalyte A containing 1% human serum albumin. The dose of cells is infused over 20-30 minutes. No leukocyte filter is used. Details of the reinfusion procedure is provided below.

The CD3+ cell dose escalates as follows: Dose level 2, 5×10^9 CD3+ cells; Dose level 3, 1×10^{10} CD3+ cells; Dose level 4, 2.5×10^{10} CD3+ cells; and Dose level 5, 5×10^{10} CD3+ cells. At all dose levels, the cells are reinfused over 20-30 minutes.

The following minimum criteria are required for release of the expanded CD3+28 T cells for reinfusion: a) Minimum cell viability of 80%; b) Less than 100 residual CD3+28 beads/3×10^6 cells reinfused; c) The bacterial, mycoplasma and fungal culture reports from day -2 must read no growth; d) The gram stain of the harvest product must report no organisms seen; and e) The endotoxin assay from day -2 must be <1.

Dose Limiting Toxicity (DLT) is a toxic event in a single patient limiting further dose escalation. For the purpose of this study, DLT is defined as any grade 3 or greater non-hematologic toxicity which is probably or definitely related to CD3+ T cells other than nausea, vomiting, or fatigue. For the purposes of this study, the Miximum Tolerated Dose (MTD) is equivalent to the dose at which at least 33% of 6 patients experience DLT. For the purposes of this study, the Recommended Phase II Dose (RPTD) is equivalent to one dose level below the MTD.

Toxicity is graded according to the CALGB Expanded Common Toxicity Criteria (Table 3). Adverse reactions not included in these Criteria are graded as follows: Mild, Grade 1; Moderate, Grade 2; Severe, Grade 3; Life-Threatening, Grade 4; and Lethal, Grade 5. Each toxic event is considered for relationship to the expanded T cells as follows: Unrelated: This category applies to those adverse events which, after careful medical consideration, are clearly felt to be due to extraneous

causes (disease, environment, *etc.*) unrelated to the administration of the expanded T cells; Unlikely: This category applies to those adverse experiences which, after careful medical consideration, are felt unlikely to be related to the administration of the expanded T cells. An adverse experience will be considered unlikely related if: 1) It does not follow a reasonable temporal sequence from administration of the expanded T cells; and 2) It could readily have been a result of the patient's clinical condition, environmental or toxic factors, or other therapies administered to the patient; Possible: This category applies to those adverse experiences which, after careful medical consideration, are felt unlikely to be related to administration of the expanded T cells but the possibility cannot be ruled out with certainty. An adverse experience can be considered possibly related if: 1) It follows a reasonable temporal sequence from administration of the expanded T cells; and 2) It could have been a result of the patient's clinical condition, environmental or toxic factors, or other therapies administered to the patient; Probable: This category applies to those adverse experiences which, after careful medical consideration, are felt with a high degree of certainty to be related to administration of the expanded T cells. An adverse experience can be considered probably related if: 1) It follows a reasonable temporal sequence from administration of the expanded T cells; and 2) It could not be reasonably explained by the patient's clinical condition, by environmental or toxic factors or by other therapies administered to the patient; Definite: This category applies to those adverse experiences which, after careful medical consideration, are felt to be related to the administration of the expanded T cells. An adverse experience can be considered definitely related if: 1) It follows a reasonable temporal sequence from administration of the expanded T cells or is associated within a period during which the expanded T cells have been established in body fluids or tissues; and 2) It could not be reasonably explained by the patient's clinical condition, by environmental or toxic factors, or by other therapies administered to the patient.

A minimum of one patient is evaluated at each dose level at which there is at most grade 0-1 toxicity. After at least one episode of grade 2 toxicity probably or definitely related to treatment (excluding alopecia, nausea/vomiting), subsequent dose

levels enroll a minimum of three patients. No more than one new patient is treated per week. The third patient entered at a dose level must be observed for at least one week prior to enrolling the first patient at the next dose level. If dose limiting toxicity occurs in one of the first three patients at a dose level, then at least three additional patients are treated at that dose level to determine if the MTD has been exceeded (fewer than three if MTD is clearly exceeded before six patients have been treated). If none of the first three patients at a dose level experience dose limiting toxicity, then dose escalation proceeds to the next level. If dose limiting toxicity occurs in more than 2 of the 6 patients, then the MTD has been reached, and the dose is decreased to define recommended phase II dose.

The toxicities related to cellular therapy with T cells are generally thought to be due to the release of cytokines from the cells. The experience with LAK cells and limited experience with CD4+ T cells suggest that the toxicity from these cells is minimal. The most common side effects are fatigue, headache, fever, nausea and chills. The more serious side effects associated with cytokines such as hypotension, capillary leak syndromes and cardiovascular collapse have not been encountered with T cell infusion therapy.

F. After Transplant Recovery

Bone marrow aspirate with biopsy for pathology review is obtained within 60 days post-reinfusion of CD34 selected PBPCs. Five to ten cubic centimeters of bone marrow aspirate is obtained in order to measure tumor cell contamination on the bone marrow mononuclear cell fraction using a PCR-based assay with tumor specific probes. CBC, Differential and platelet count are performed every other week. Chem-17 is performed every other week. Twenty cubic centimeters of heparinized peripheral blood is collected on the same day as the post-transplant bone marrow specimen, then every 30 days for the first three months, every 60 days for the next three months, followed by every 90 days for six months or for a minimum of 18 months after reinfusion of CD34+ cells. Tumor cell contamination is determined on the peripheral blood mononuclear cell fraction using a PCR-based assay with tumor

specific probes. Tumor reevaluation is performed every three months with CT and SPECT-gallium scans for one year following therapy.

Complete Response (CR) is defined as no clinical, radiologic, or histopathologic evidence (marrow evaluation) of tumor for 6 or more months duration. Partial Response (PR) is defined as a minimum of 50% shrinkage in tumor with no evidence of progression at any site and no new sites of disease. Patients not meeting the criteria for CR or PR are considered to have No Response (NR). Progressive disease (PD) is defined as > 25% increase in size of an existing lesion or the appearance of any new lesion. Survival time is calculated from CD34 selected PBPC reinfusion (Day 0) until death or last patient contact. Time to Progression (TTP) and Time to Treatment Failure (TTF) are calculated from reinfusion until disease progression or last patient contact, and from reinfusion until any event defining treatment failure (PD, death from any cause, second malignancy, *etc.*) respectively.

TABLE 3

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
HEMATOLOGIC	0	1	2	3	4
WBC	≥4.0	3.0-3.9	2.0-2.9	1.0-1.9	<1.0
PLT	WNL	75.0-normal	50.0-74.9	25.0-49.9	<25.0
Hgb	WNL	10. 0-normal	8.0-10.0	6.5-7.9	<6.5
Granulocytes/ Bands	≥2.0	1.5-1.9	1.0-1.4	0.5-0.9	<0.5
Lymphocytes	≥2.0	1.5-1.9	1.0-1.4	0.5-0.9	<0.5
Hematologic-Other		mild	moderate	severe	life-threatening
HEMORRHAGE (clinical)	none	mild, no transfusion	gross, 1-2 units transfusion per episode	gross, 2-4 units transfusion per episode	massive, >4 units transfusion per episode
INFECTION	none	mild no active treatment,(*e.g.*, viral syndromes	moderate requires outpatient PO antibiotic	severe requires IV antibiotic or antifungal or hospitalization	life-threatening (*e.g.*, septic shock)
GABTROINTESTINAL					
Nausea	none	able to eat reasonable intake	intake significantly decreased but can eat	no significant intake	-----

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
Vomiting	none	1 episode in 24 hrs	2-5 episodes in 24 hrs	6-10 episodes in 24 hrs	>10 episodes in 24 hrs or requiring parenteral support
Diarrhea	none	increase of 2-3 stools/day over pre Rx	increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	increase of 7-9 stools/day, or incontinence, or severe cramping	increase of ≥10 stools/day or grossly bloody diarrhea, or need for parenteral support
Stomatitis	none	painless ulcers, erythema, or mild soreness	painful erythema, edema, or ulcers, but can eat	painful erythema, edema, ulcers, and cannot eat	requires parenteral or enteral support
Esophagitis/Dysphagia	none	painless ulcers, erythema, mild soreness, or mild dysphagia	painful erythema, edema, or ulcers or moderate dysphagia but can eat without narcotics	cannot eat solids or requires narcotics to eat	requires parenteral or enteral support or complete obstruction or perforation
Anorexia	none	mild	moderate	severe	life-threatening

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA					
			Grade		
TOXICITY	0	1	2	3	4
Gastritis/Ulcer	no	antacid	requires vigorous medical management or non-surgical treatment	uncontrolled by medical management; requires surgery for GI ulceration	perforation or bleeding
Small Bowel Obstruction	no		intermittent, no intervention	requires intervention	requires operation
Intestinal Fistula	no	-----	-----	yes	-----
GI-other	-----	mild	moderate	severe	life-threatening
OTHER MUCOSAL	none	erthema, or mild pain not requiring treatment	patchy & produces serosanguinous discharge or requires non narcotic for pain	confluent fibrinous mucositis or requires narcotic for pain or ulceration	necrosis
LIVER					
Bilirubin	WNL	-----	1.5xN	1.5-3.0xN	>3.0xN
Transaminase (SGOT. SGPT)	WNL	≥2.5xN	2.6-5.0xN	5.1-20.0xN	>20.0xN

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
Alk Phos or 5'nucleotidase	WNL	≥2.5xN	2.6-5.0xN	5.1-20.0xN	>20.0xN
Liver-clinical	no change from baseline	-----	-----	precoma	hepatic coma
Liver-other	-----	mild	moderate	severe	life-threatening
KIDNEY, BLADDER					
Creatinine	WNL	<1.5N	1.5-3.0xN	3.1-6.0xN	>6.0xN
Proteinuria	no change	1+or< 0.3-1g% or <3 g/l	2-3+ or 0.3-1.0 g% or 3-10 g/l	4+ or > 1.0 g% or >10 g/l	nephrotic syndrome
Hematuria	neg	micro only	gross, no clots	gross + clots	requires transfusion
BUN mg%	WNL, <20	21-30	31-50	>50	-----
Hemorrhagic Cystitis	none	blood on microscope exam	frank blood no treatment required	bladder irrigation required	requires cystectomy or transfusion
Renal Failure	-----	-----	-----	-----	dialysis required
Incontinence	normal	with coughing sneezing, *etc.*	spontaneous some control	no control	-----

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
Dysuria	none	mild pain	painful or burning urination, controlled by pyridium	not controlled by pyridium	-----
Urinary Retention	none	urinary residual >100 cc or occasionally requires catheter or difficulty initiating urinary stream	self catheterization always required for voiding	surgical procedure required (TUR or dilation)	-----
Increased Frequency/Urgency	no change	increase in frequency of nocturia up to 2x normal	increase > 2x, but<hourly	with urgency and hourly or more or requires catheter	-----
Bladder Cramps	none	-----	yes	-----	-----
Ureteral Obstruction	none	unilateral, no surgery required	bilateral, no surgery required	not complete bilateral, but stents, nephrostomy tubes or surgery required	complete bilateral obstruction
GU Fistula	none	-----	-----	yes	-----
Kidney/Bladder-Other	-----	mild	moderate	severe	life-threatening

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
ALOPECIA	no loss	mild hair loss	pronounced or total hair loss	-----	-----
PULMONARY					
Dyspnea	none or no change	asymptomatic, with abnormality in PFTs	dyspnea on significant exertion	dyspnea at normal level of activity	dyspnea at rest
pO2/pCO2	no change of pO2>85 and pCO2≤40	pO2>70 and pCO2≤50, but not grade 0	pO2>60 and pCO2≤60 but not grade 0-1	pO2>50 and pCO2≤70 but not grade 0-2	pO2≤50 or pCO2>70
Carbon Monoxide Diffusion Capacity (DLCO)	>90% o pretreatment value	decrease to 76-90% of pretreatment	decrease to 51-75% pretreatment	decrease to 26-50% pretreatment	decrease to ≤25% of pretreatment
Pulmonary Fibrosis	normal	radiolographic changes, no symptoms	-----	changes with symptoms	-----.
Pulmonary Edema	none	-----	-----	radiographic changes and diuretics	requires intubation

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
Pneumonitis (non infectious)	Normal	Radiographic changes symptoms do not require steroids	steroids required	oxygen required	requires assisted ventilation
Pleural Effusion	none	present	-----	-----	-----
Adult Respiratory Distress Syndrome	none	mild	moderate	severe	life-threatening
Other Pulmonary: Cough	no change	mild, relieved by OTC meds	requires narcotic antitussive	uncontrolled coughing spasms	.....
Pulmonary-Other		mild	moderate	severe	life-threatening
HEART					
Cardiac Dysrhythmias	none	asymptomatic. transient, requiring no therapy	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension, or ventricular tachycardia. or fibrillation

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
Cardiac Function	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	severe or refractory CHF
Cardiac Ischemia	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic. decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	sever or refractory CHF
Cardiac-pericardial	none	asymptomatic, effusion, no intervention required	pericarditis (rub, chest pain, ECG changes)	symptomatic effusion; drainage required	tamponade; drainage urgently required
Heart-Other		mild	moderate	severe	life-threatening
CIRCULATORY					

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

TOXICITY	Grade 0	1	2	3	4
Hypertension	none or no change	asymptomatic. transient increase by greater than 20 mm Hg(D) or to >150/100 if previously WNL. No treatment required.	recurrent or persistent increase by greater than 20 mm Hg (D) or to >150/100 if previously WNL. No treatment required	requires therapy	hypertensive crisis
Hypotension	none or no change	changes requiring no therapy (including transient orthostatic hypotension)	requires fluid replacement or other therapy but not hospitalization	requires therapy and hospitalization; resolves within 48 hrs of stopping the agent	requires therapy and hospitalization for >48 hrs after the stopping agent
Phlebitis/Thrombosis/ Embolism			superficial phlebitis (not local)	Deep vein thrombosis	major event (cerebral/hepatic/ pulmonary/other infarction) or pulmonary embolism
Edema	none	1+ or dependent in evening only	2+ or dependent throughout day	3+	4+, generalized anasarca

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA					
	Grade				
TOXICITY	0	1	2	3	4
NEUROLOGIC					
Neuro-sensory	none or no change	mild paresthesias, loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paresthesias	severe objective, sensory loss or paresthesias that interfere with function	-----
	none or no change	subjective weakness; no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neuro-cortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe: somnolence or agitation or confusion or disorientation, or hallucinations or aphasia, or severe difficulty communicating	coma, seizures, toxic psychosis
Neuro-cerebellar	none	slight incoordination dysdiadokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor staxia	cerebellar necrosis

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

TOXICITY	Grade 0	1	2	3	4
Neuro-mood	anxiety-none depression none	mild anxiety mild depression	moderate anxiety moderate depression	severe anxiety severe depression	severe agitation suicidal ideation
Neuro-headache	none	mild	moderate or severe but transient	unrelenting and severe	-----
Neuro-constipation	none or no change	mild	moderate	severe	ileus >96 hrs
Neuro-hearing	none or no change	asymptomatic. hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro-vision	none or no change	-----	-----	symptomatic subtotal loss of vision	blindness
Pain	none	mild	moderate	severe	intolerable
Behavioral Change	no change	change, not disruptive to pt. or family	disruptive to pt. or family	harmful to others or self	psychotic behavior

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

TOXICITY	Grade 0	1	2	3	4
DizzinessNertigo	none	non-disabling	-----	disabling	-----
Taste	normal	slightly altered taste, metallic taste	markedly altered taste	-----	-----
Insomnia	normal	occasional difficulty sleeping may require sleeping pills	-----	difficulty sleeping despite medication	-----
Neurologic Other		mild	moderate	severe	life-threatening
DERMATOLOGIC					
Skin	none or no change	scattered macular or papular eruption or erythema that is asymptomatic	scattered macular or papular eruption or erythema with pruritus or other associated symptoms	generalized symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Local	none	pain	pain and swelling with inflammation or phlebitis	ulceration	plastic surgery indicated

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

TOXICITY	Grade 0	1	2	3	4
ALLERGY	none	transient rash, drug fever <38C, 100.4F	urticaria, drug fever ≥38C 100.4F, mild bronchospasm	serum sickness, bronchospasm, req. parenteral meds	anaphylaxis
FLU-LIKE SYMPTOMS					
Fever in absence of infection	none	37.1-38.0C 98.7-100.4F	38.1 C-40.0C 100.5-104,0F	>40.0C >104.0F for less than 24 hrs	>40.0C (104.0F) for more than 24 hrs or fever accompanied by hypotension
Chills	none	mild or brief	pronounced or prolonged	-----	-----
Myalgia/Arthralgia	normal	mild	decrease in ability to move	disabled	-----
Sweats	normal	mild and occasional	frequent or drenching	-----	-----
Malaise/Fatigue*	none	mild, able to continue normal activities (PS1)	impairment of normal daily activity or bed rest <50% of waking hours (PS2)	in bed or in chair >50% of waking hours (PS3)	bed ridden or unable to care for self (PS4)

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
Flu-Like Symptoms-Other	-----	mild	moderate	severe	life-threatening
WEIGHT GAIN	<5.0%	5.0-9.90%	10.0-19.9%	>20%	-----
WEIGHT LOSS	<5.0	5.0-9.9%	10.0-19.9%	>20%	-----
METABOLIC					
Hyperglycemia	<116	116-160	161-250	251-500	>500 or ketoacidosis
Hypoglycemia	>64	55-64	40-54	30-39	<30
Amylase	WNL	<1.5xN	1.5-2.0xN	2.1-5.0xN	>5.1 xN
Hypercalcemia	<10.6	10.6-11.5	11.6-12.5	12.6-13.5	≥13.5
Hypocalcemia	>8.4	8.4-7.8	7.7-7.0	6.9-6.1	≤6.0
Hypomagnesemia	>1.4	1.4-1.2	1.1-0.9	0.8-0.6	≤0.5
Hyponatremia	no change or >135	131-135	126-130	121-125	≤120
Hypokalemia	no change or >3.5	3.1-3.5	2.6-3.0	2.1-2.5	≤2.0
Metabolic-Other		mild	moderate	severe	life-threatening

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA

	Grade				
TOXICITY	0	1	2	3	4
COAGULATION					
Fibrinogen	WNL	0.99-0.75xN	0.74-0.50xN	0.49-0.25xN	≥0.24xN
ProthrombinTime	WNL	1.01-1.25xN	1.26-1.50xN	1.51 -2.00xN	>2.00xN
Partial thromboplastin time	WNL	1.01-1.66xN	1.67-2.33xN	2.34-3.00xN	>3.00xN
Coagulation	-----	mild	moderate	severe	life-threatening
Impotence/Libido	normal	decrease in normal function	-----	absence of function	-----
Sterility	-----	-----	-----	yes	-----
Amenorrhea	no	yes	-----	-----	-----
Gynecomastia	normal	mild	pronounced or painful	-----	-----
Hot flashes	none	mild or <1/day	moderate and ≥1/day	frequent and interferes with normal function	-----
Cushingold	normal	mild	pronounced	-----	-----
Endocrine Other		mild	moderate	severe	life-threatening
EYE					

TABLE 3 - Continued

CAL GR EXPANDED COMMON TOXICITY CRITERIA					
			Grade		
TOXICITY	0	1	2	3	4
Conjunctivitis/Keratitis	none	erythema or chemosis not requiring steroids or antibiotics	requires treatment with steroids or antibiotics	corneal ulceration or visible opacification	-----
Dry Eye	normal	-----	requires artificial tears	-----	requires enucleation
Glaucoma	no change	-----	-----	yes	-----
Eye Other	-----	mild	moderate	severe	life-threatening

EXAMPLE 5

Mononuclear Cell Collection

Mononuclear cells are collected using the 1-120 stem cell program on the Baxter Fenwal CS3000 Plus Blood Cell Separator. The CS3000 Plus Blood Cell Separator is a self-contained, continuous flow, centrifugal device that separates whole blood into some of its components. Collection of specific blood components can be automatically implemented and monitored. A program for each type of collection procedure is stored in the solid state memory of the separator. Two operating modes are provided: automatic and manual. During automatic operation, all functions are controlled by a microcomputer except during situations when operator intervention is required. During manual operation, all functions are under control of the operator. A message center on the operator panel provides operator help messages and status messages, and also allows the operator to program the separator to perform special procedures. Blood components are collected in a sterile, disposable Apheresis Kit. The components are centrifugally separated within the Apheresis Kit by density differences. A two-stage centrifugation process is used in most procedures.

Various monitoring systems are continuously checked for abnormal or potentially hazardous conditions. Digital readouts, indicator LED's and audible alarms keep the operator informed of status and abnormal conditions. If appropriate action is not taken to correct an abnormal condition within a specified amount of time, the separator halts the procedure and isolates the donor from the system. An alphanumeric message center provides status messages, alarm information and help messages throughout the separation procedure.

The centrifuge consists of two primary mechanical components: the motor driven rotor and the shield assembly. The rotor consists of a yoke which supports two diametrically opposed clamp assemblies. Each clamp assembly supports a container holder that secures one of the flexible plastic containers (separation or collection) of the Apheresis Kit. Each container holder consists of a plastic insert and metal plate

which together form a cavity of a specific configuration. When the associated plastic container of the kit is clamped between the insert and plate, the container takes the shape of the cavity. The separation container holder is used to harvest white cells. The small volume collection container (SVCC) is used to collect the mononuclear cells.

During operation, anticoagulated whole blood is pumped into the spinning separation container by the whole blood pump (WB/ACD pump). The primary stage of separation takes place in the separation container, which is analogous to the "first spin" operation of a blood component collection process. As the whole blood enters the separation container, the higher density blood components, primarily RBC's, are packed by centrifugal force toward the outer edges of the container. These heavier components then exit the separation container *via* the PRBC (packed red blood cells) line. The lower density components such as plasma, platelets and white cells can then be extracted by the plasma pump via the CRP (component-rich plasma) line. The CRP is pumped into the collection container where the secondary stage of component separation takes place, which is analogous to the "second spin" of a blood component collection process. As the CRP enters the bottom of the collection container, the heavier component (white cells) is packed by centrifugal force against the back wall of the container. The separated components remain packed within the container while the lower density components (plasma and platelets) exit the container via the CPP (component-poor plasma) line. The CPP can then be recombined with the PRBC's and returned to the donor or collected in one of the Transfer Pack containers of the Apheresis Kit.

The 1-120 Stem Cell Program is used in this procedure for the collection of mononuclear cells (stored under Special Procedure I in the separator's memory). Mononuclear cells are those cells with one nucleus and are collected because the targeted cells are found in this fraction of leukocytes. The 1-120 Program is slightly different from the original 1-100 Program. The 1-120 Program includes several changes to reduce the blocked line alarms at spillover. It permits plasma flow rate

settings according to the donor's hematocrit and permits optical settings according to the absolute mononuclear cell count of the donor. This program can be used at draw rates between 20 and 80 ml/min by changing the draw rate using the front panel keys in the same method used in platelet apheresis. No program changes are made when changing draw rates.

A. Programming The Memory of the 1-120 Program Separator

Turn the power on to the CS3000 by depressing the power button on the front panel, and verify that the power on self-test is successful. Then close the master reset control knob by turning it clockwise until "pre-prime" is displayed in the message center. Procedure 8 (Baseline Cell Collection) is selected using the procedure select key, and the edit key is pressed on the manual control panel. The table edit feature allows the operator to alter the preset procedures which are stored as tables in the separator's memory and to store the altered version of the tables for future use. These tables govern the method used to control the pumps speed, centrifuge speed, end point, interface detector offset, clamps settings and algorithms used to compute the plasma pump speed during the automatic mode of operation.

The desired table to edit (the run table) is selected using either the location or contents keys. The prime table is left unaltered as the prime cycle is identical for all procedures. Press the edit key to edit the run table: the up/down location arrow keys are used to change locations, and the up/down content arrow keys are used to change contents. New values appear. Enter the content values on Table 4 shown below for each location as specified. Repeat using the up/down arrow keys until all desired location changes are entered. Press the store key to save the location changes.

Then select special procedure 1 for storage by using the up/down arrow keys. Press the store key to store the new content values in the permanent memory of special procedure 1. Select special procedure 1 using the procedure select key. Repeat the steps above to check the locations for the correct contents. This step is to verify that the values from Table 4 previously entered were properly stored into the

permanent memory of special procedure 1. If any errors are noted, the correct contents are reprogrammed using the up/down content arrows, and the correct procedure is stored while in special 1. If all contents are correct, press edit to exit.

TABLE 4

LOCATION	VALUE	LOCATION	VALUE	LOCATION	VALUE
00	1	30	1	60	175
01	14	31	42	61	0
02	77	32	77	62	16
03	30	33	100	63	0
04	150	34	0	64	36
05	1	35	1	65	0
06	10	36	12	66	0
07	0	37	0	67	0
08	64	38	0	68	55 (see Table 5)
09	2	39	0	69	0
10	1	40	0	70	2
11	14	41	0	71	120 (see Table 6)
12	77	42	0	72	255
13	30	43	0	73	102
14	150	44	0	74	92
15	1	45	0	75	50
16	11	46	0	76	81
17	0	47	0	77	28
18	0	48	0	78	90
19	0	49	0	79	29
20	0	50	0	80	251
21	14	51	0	81	5
22	77	52	0	82	5
23	30	53	0	83	50
24	150	54	0	84	146

TABLE 4 - Continued

LOCATION	VALUE	LOCATION	VALUE	LOCATION	VALUE
25	1	55	0	85	16
26	3	56	0	86	39
27	0	57	0	87	4
28	0	58	0	88	16
29	0	59	0	89	0

TABLE 5

Location 68 Set Per Donor Hematocrit As Follows

HEMATOCRIT	LOCATION VALUE
20 - 25	70
26 - 30	65
31 - 35	60
36 - 40	55
41 - 45	50
46 - 50	45

TABLE 6

Location 71 Set Per Donor Mononuclear Cell (MNC) Count As Follows

MNC COUNT	LOCATION VALUE
BELOW 500/μL	100
500 - 3000/μL	120
3000 - 5000/μL	140
5000/μL AND ABOVE	160

The MNC (mononuclear cell) count is the absolute lymphocyte count plus the absolute monocyte count per microliter. It is calculated by multiplying the donor's WBC count by the sum of the percentages of lymphocytes and monocytes. For example, if the donor's CBC reflects a WBC count of 7400 μL with 33%

lymphocytes, 4% monocytes, 63% granulocytes and a HCT of 39%, the MNC count is calculated as: $7400 \times (.33 + .04) = 2738$. Accordingly, the donor's absolute MNC count is 2738/μL. The interface setting (Location 71) is 120.

It is important to accurately calculate the MNC count of the immunocompromised donor and adjust the value of Location 71 as appropriate. During the MNC collection process described herein, the value of Location 71 is routinely programmed as 90 to reduce the amount of erythrocyte and granulocyte contamination in the final product. In general, Location 71 should always have a value of 90. Table 6 is provided to set guidelines for the programming of Location 71 should the Interface Optic Detector be set according to the donor's MNC count.

Then select special procedure 1 using the procedure select key. Press the edit key on the manual control panel. Select the reinfuse table using either the location or contents keys. Program the location values in Table 7 into the reinfuse table.

TABLE 7

LOCATION	VALUE	LOCATION	VALUE	LOCATION	VALUE
00	1	20	1	40	0
01	1	21	1	41	0
02	26	22	26	42	0
03	100	23	100	43	0
04	0	24	0	44	0
05	223	25	223	45	0
06	10	26	150	46	0
07	0	27	0	47	0
08	0	28	0	48	0
09	0	29	0	49	0
10	1	30	1	50	0
11	1	31	0	51	0
12	26	32	40	52	0

TABLE 7 - Continued

LOCATION	VALUE	LOCATION	VALUE	LOCATION	VALUE
13	100	33	0	53	0
14	95	34	0	54	0
15	223	35	0	55	0
16	5	36	100	56	0
17	0	37	0	57	0
18	0	38	0	58	0
19	0	39	0	59	0

Repeat the verification process as described previously to ensure that the values from Table 7 that were programmed into the reinfuse table of special procedure 1 have been properly stored into the permanent memory.

Next, the Closed System Apheresis Kit is installed on the CS3000 Plus Separator. Ensure that the Separation Chamber is installed in the separation container holder clamp assembly and that the SVCC is installed in the collection container holder clamp assembly. Aside from the changes specified above, all other steps for priming the Closed System Apheresis Kit are unaltered.

B. Mononuclear Cell Collection

Ensure that the auto/manual run is set to auto (auto led on) and that special 1 is displayed in the message center. Press the display/edit key on the front panel. Reduce the end point volume to 5000 ml by pressing the enter key. The message center displays the current setting (10000) and the new setting. Press the double down arrow key until 5000 is displayed under "New". Press the enter key once again to enter the new setting. The end point is routinely decreased to 5000 ml.

Press the single up arrow key to move to the next parameter which is whole blood flow rate. The default for the blood flow rate is 50 ml/min. It is advisable to

leave this parameter unchanged until the first Spillover occurs and the blood flow from the Inlet Needle is proven to be appropriate. Move to the next parameter by pressing the single up arrow key. The plasma flow rate is left unchanged as the separator's computer determines this parameter according to the location contents programmed into the memory. The centrifuge speed parameter should be 1600. The interface detector offset should be set to 90. If a parameter other than 90 is displayed, press the enter key, and using the up or down arrow keys, select 90 and press the enter key again.

The interface detector baseline parameter is set by the computer's memory automatically, and should be left unaltered. The plasma volume (Coll/Exch) is set to 200. Press the enter key once, the double up arrow key twice to select 200 and the enter key again. By setting this parameter to 200, the CS3000 Plus Separator pumps 200 ml of the prime into the 600 ml Transfer Pack of the Apheresis Kit at the beginning of the run. During the prime cycle, the Apheresis Kit is filled with approximately 300 ml of priming solution, about 100 ml of this volume is ACDA anticoagulant. By diverting 200 ml of the prime solution to the Transfer Pack, the possibility of causing early signs of citrate toxicity in the donor is reduced. Unless extracorporeal volume is a concern, the standard setting for the plasma volume parameter is 200.

The single access cycle volume and the transfer pack volume parameters are unaltered. The default for both is 0. These parameters are used only when performing a one-arm procedure. To accept the parameters entered and return to the prerun screen, press the display/edit key. Make sure that the drip chambers of the Saline and Anticoagulant containers are at least 1/2 full but no more than 3/4 full to facilitate determination of drip rates and to prevent air from entering the system.

Prime the Return Line to the "Y" junction, and open the Roberts clamp to fill Sampling Pack with sufficient blood to collect the required samples. Close the Roberts clamp, and heat seal the Sampling pack twice. Open the Return Line roller

clamp to flush the line. Control the Saline flow rate with the Return Line roller clamp and maintain the site at KVO rate. Collect the required blood samples from the Sampling Pack using a Vacutainer needle and holder. The Sampling Pack may be detached from the Return Line or left connected. Prime the Inlet Line just to the tip of the needle. Press the mode key to select run. With run indicated in the message center, press the start/resume key twice to initiate the Auto Run. Immediately open the Inlet Line roller clamp and the Return Line roller clamp. The centrifuge and pumps start immediately.

Determine the anticoagulant flow rate by monitoring the drip rate in the ACD drip chamber of the Apheresis kit, and record the flow rate. To achieve the best possible separation, a WB:ACD ratio of 10:1 is recommended unless the donor is highly sensitive to the anticoagulant or symptoms of hypocalcemia are reported by the donor. See Table 8 below to determine WB:ACD ratios according to drip rate.

TABLE 8

ANTICOAGULANT DROP COUNT GUIDE

WBFR (ml/min)	9:1 (~drops/min)	10:1 (~drops/min)	11:1 (~drops/min)	13:1 (~drops/min)
70	90	83	74	64
60	77	71	64	55
50	64	59	53	45
40	51	48	43	36

Ensure that the roller clamps on the Saline and Vent lines are completely open. This allows free flow of Saline solution whenever the halt/irrigate key is depressed. Within 5 to 10 minutes, the first Spillover occurs and message 80 (Spillover Steps in Progress) is displayed in the message center. Once the Spillover steps are over, message 89 (Run in Progress) reappears. At this time, increase the Whole Blood Flow Rate to 60 ml/min if blood flow from the donor is appropriate, and press the display/edit key. Using the single up/down arrow keys, select the whole blood flow

rate screen. Press the enter key and using the double or single up arrow keys, increase the value to 60. Press the enter key once again to enter the change. The single up/down arrow keys changes the value by ones, and the double up/down arrow keys changes the value by 10s.

The WBFR value is now 60. Press the display/edit key to accept and implement the change entered. The blood (ml/min) display on the front panel should reflect the change in WBFR. After the plasma flow rate stabilizes, spillovers occur approximately every 3.5 minutes throughout the procedure. Every 15 minutes, the volume processed, the whole blood pump flow rate, the plasma pump flow rate, the anticoagulant drip rate, the centrifuge speed and the return plasma clarity are checked and recorded. The separated plasma in the Plasma Return Line is visually inspected for hemolysis. A red tinge to the plasma in the Return Line is cause for evaluation (prior to reinfusion) to determine if this is from hemolysis or red cell contamination of the separated plasma.

After 4100 ml of blood have been processed, message 61 (Check Anticoagulant Container) alerts the operator to ensure that sufficient anticoagulant remains in the container to complete the procedure. To silence the alarm, clear the message and continue with the apheresis procedure, press the start/resume key once. The Auto Run continues until the end point is reached. If it is desired to terminate the procedure before the end point is reached, press the halt/irrigate key once. At this time, the mode and start/resume LEDs flash. Press the mode key to select Reinfuse. Then press the start/resume key to initiate the Reinfuse operation. If it is desired to pause the procedure for any reason and later restart at the same point where paused, press the halt/irrigate key. Once the reason for pausing has been resolved, press the start/resume key to continue with the procedure.

When the end point is reached (blood volume display equals or exceeds the end point display), message 60 (End Point Reached) is displayed, a one note chime sounds and the mode and start/resume LEDs flash. To discontinue the Run procedure

and initiate the Reinfuse operation, press the mode key to select Reinfuse. Then press the start/resume key to initiate the Reinfuse operation. To continue the procedure, select a new end point using the display/edit function and then press the start/resume key. If message 80 (Spillover Steps in Progress) is displayed in the message center at the moment that the end point is reached, message 60 is not displayed until the Spillover Steps are completed. Do not press the halt/irrigate key until message 60 appears to prevent compromising the purity of the final product.

While the Reinfuse operation is in progress, close the Inlet Line roller clamp, heat seal the line twice and separate from the Apheresis Kit.. Remove the Inlet needle from the donor and dispose in a sharps container. After applying pressure on the phlebotomy site for approximately three minutes, using a sterile 2×2 cohesive bandage, wrap the donor's arm. Instruct the donor to refrain from moving the arm with the Return needle as fluid is being infused into that arm and infiltration may occur. When the Reinfuse operation is complete, message 25 (Procedure Complete) is displayed and a one note chime sounds. Press the mute key to silence the chime, close the Return Line roller Clamp, heat seal the line twice and separate from the Apheresis Kit. Remove the Return needle from the donor's arm and dispose in a sharps container. Apply pressure to the phlebotomy site and bandage the arm as explained above. The donor is provided refreshments and both verbal and written post-donation instructions. The donor's vital signs are checked and recorded. Close all the roller clamps on the Apheresis Kit. Then the end time, total volume of blood processed, donor's post donation vital signs, amount of Saline and anticoagulant used and how well the procedure was tolerated by the donor are recorded.

C. Transfer Of The Collected Cells

Once the roller clamps on the Apheresis Kit are closed, the doors to the centrifuge compartment of the separator are opened and the multiple lumen tubing is removed from the upper support bar and the lower restraining collar. Open the separation and collection container holders and remove the collection and separation containers. Resuspend the cells in the collection container by gentle agitation for 3 to

5 minutes or until aggregates are no longer visible. Press the start/resume key to open the Plasma Collect clamp and remove the tubing from the clamp. Remove the 600 ml transfer pack and the two 1000 ml PL732 containers from their hook. Temporarily invert the PL732 containers, open their roller clamps and transfer the air from the containers into the collection container by squeezing to expel the air. Before releasing the PL732 containers, close their roller clamps. Place the PL732 containers and the 600 ml transfer pack in the bottom of the centrifuge compartment. Hang the collection container from the WBIACD pump handle. Open the roller clamp on one of the PL732 containers and allow all the cells in the collection container to drain into this container. Once all the cells have drained into the PL732 container, squeeze the empty collection container to expel the air into the PL732 container. Close the roller clamp to the PL732 container that now contains the cells. This action clears the lines of any residual cells and facilitates the passage of the collection and separation containers through the guide hole of the rotor assembly during removal of the Apheresis Kit from the separator. Roll the PL732 container with the cells to push excess air toward the ports of the container. Open the roller clamp on this container and purge excess air into the Component Poor Plasma line leaving some air in the container to facilitate proper emptying of the container during further processing. Close the roller clamp to this container as close to the ports as possible.

Heat seal the tubing leading from the PL732 container with cells to the manifold three times. Cut between the seals leaving two seals on the tubing of the PL732 container with cells. Weigh the cells container and record the weight. Any fluid leakage or Apheresis Kit disconnection constitutes a break in sterility and the set must be discarded and replaced. If this happens during the priming procedure, simply discard the faulty Apheresis Kit and install a new one. Should the break in sterility occur during the actual collection process, and not inside the centrifuge compartment of the cell separator, press the halt/irrigate key and immediately close both the Inlet and Return Line roller clamps, determine the cause for loss of sterility and contain any spill of fluid, explain the situation to the donor, and do not perform a Reinfuse operation. Remove the Apheresis Kit and dispose appropriately.

Should the break in sterility occur inside the centrifuge compartment, the spill of fluid triggers a Code 42 alarm. This alarm condition shuts down all the separator's functions. Press the mute key to silence the audible alarm and close the roller clamps of the Inlet and Return Lines. Again, determine the cause of loss of sterility and do not perform a Reinfuse operation.

If the starting plasma flow rate is set according to the donor's Hematocrit, the CS3000 begins collecting MNCs sooner and consequently has an overall higher efficiency of collection. While the computer selects the appropriate setting during the first liter processed, pre-setting the plasma flow rate speeds the process and improves yield by a few percent.

The use of the SVCC (Small Volume Collection Chamber) reduces the amount of platelet contamination of the final product and reduces the extracorporeal volume. While the final volume of the SVCC is approximately 50 ml, it contains about 25 ml of cells and requires about 25 ml of space for plasma. About 2 ml of red and white cells are concentrated from donors with up to 3000 MNCs/μL for each liter of whole blood processed. The 1-120 Program collects an average of 60% +/- 25% of the MNCs that are processed, although the collection efficiency is very donor dependent. ACD is important for cell collection. The ideal ratio of WB:ACD is 10:1, which means using 100 ml of ACD per 1000 ml of whole blood processed. If not enough ACD is used, it may cause the cells to clump and not separate finely for a pure collection.

EXAMPLE 6

Autologous Stem Cell Rescue

Autologous stem cell rescue (ASCR) is used to restore hematopoietic function following high-dose chemotherapy-radiotherapy. There are many sources of stem cells including bone marrow, peripheral blood, and umbilical cord blood. During the

interval between cell collection and reinfusion, the cells' metabolic 'clock' should completely halt. Successful 'freezing' or cryopreservation of stem cells prohibits further metabolism while maximizing viability. The key aspects include use of a cryoprotective agent such as dimethyl sulfoxide (DMSO) and a controlled rate of cooling.

Cells are easily damaged through rapid or slow rates of cooling. Dimethyl sulfoxide protects cells from damage due to slow cooling, while a controlled rate guards against rapid cooling. This is especially true at the most critical point when cells undergo phase change from a liquid to a solid. At this heat of fusion point, cells give off energy in the form of heat which is destructive to them unless the heat is compensated for by additional cooling. Excessive cooling, however, is also lethal to these sensitive cells. The following protocol was developed for a variable heat of fusion point, using the actual specimen rather than a 'dummy' bag for monitoring the rate of cooling. Following cryopreservation, the cells are immediately stored in liquid phase of nitrogen where they may remain for several days, months, or many years without significant degeneration.

The DMSO-Medium solution should be made on the same day as the cryopreservation. The final concentration of DMSO when DMSO-medium is mixed with cells in a 1:1 proportion is 10%. Hence, the initial concentration of DMSO in medium must be twice that, or 20%. To determine the total amount of DMSO and medium needed, weigh the specimen. The weight approximates the specimen's volume for which an equal volume of DMSO-medium solution is required. In addition, a few extra milliliters (ml) for cryovials is needed.

Place Tissue Culture medium, Dimethyl Sulfoxide (Cryoserv; Research Industries Corporation, Salt Lake City, Utah), two sterile 50 cc conical tubes (any sterile container is appropriate), one 18G 1.5" sterile hypodermic needle, GIBCO Medium 199 with Hank's salts and L-glutamine, (GIBCO Laboratories, Grand Island, NY), 1 sterile 25 or 50 ml pipet,; a pipet-aid (Drummond Sci. Co., Broomall, PA), a

metal or plastic rack for conical tubes, sterile alcohol prep pads, and 1 sterile syringe under a laminar flow hood. Remove the plastic wrap from bottle of medium and loosen cap. Pour medium carefully into each of two sterile 50 cc tubes. Pipet appropriate volume back into plastic bottle and re-cap loosely. If attached, remove metal cap from Cryoserv with an alcohol prep pad. Wipe rubber injection site with alcohol. Attach 18G 1.5" needle to syringe, insert the needle and aspirate the DMSO. Inject DMSO into medium, cap tightly, shake well (outside of hood), and chill in refrigerator at 4°C.

Turn on the computer and recorder. Remove the protective cap from the recorder pen and 'zero' accordingly. Use "adv" to see pen position. On the computer, press in sequence, "cool", "cool +", "fan", "alarm", and "run" keys, and lastly, press number 1 if using program one. Close the chamber door, and place a Styrofoam blood tube box near freezer.

A. Preparing The Specimen

The metal holders should be labeled with unique number, name, specimen type, date of birth, cryopreservation date and any other pertinent information such as hospital chart number. A "holder", "cassette" or "canister" are all metal plates used to press the specimen into an even layer and protect the frozen cryobag. The Fenwal cryobags fit imperfectly but adequately in the metal holders custom-made by Stealcon, which in turn, fit into frames made by Custom Biogenic Systems, for storage in liquid nitrogen (Stericon, Broadview, IL). Cryobags require similar information plus institution. Do not write directly on the cryobag plastic because the ink may diffuse into the bag. Instead write on the upper left and right corners of cryobags, or use cryobags with pockets. To ascertain the same freeze rate, each bag should contain the same volume. Chill holders while awaiting the specimen.

Next to the hood, the Sebra sealer should be turned on. Under the hood place with your specimen: n cryobags where n = the final number of cryobags; 1 blood component infusion set (bcis); (n + 1) 60 cc syringes; one 10 cc syringe; sterile

conical tube(s) including one labeled "immix"; a metal or plastic tube rack; a Pipet-Aid; 1 sterile 2 ml pipet; and 2 sterile 1 or 2 ml pipets (depends on cryovial number and volume).

Close all clamps on cryobags. Remove bcis from box and close all clamps. Remove cover from the specimen bag outlet port and without touching port, hold or set bag aside. Insert bcis spike into outlet pod of specimen bag. Open 10 cc syringe and set aside. Remove cover from bcis female Luer connector and attach the 10 cc syringe. Open clamp to specimen bag and withdraw pre-determined amount for cryovials, CD 34+ testing, *etc.*, then close clamp and lay down. Open a 60 cc syringe and set aside then detach a 10 cc syringe and wedge between thumb and remaining fingers in cusp of hand, and attach 60 cc syringe to bcis female Luer.

There are four different sizes of cryobags called Cryocyte Freezing Containers. The plastic is PL269. Total volume in the 500 ml sized bag, Code 4R5462 (Changed to 4R9954), should not exceed 110 ml/because the bag may be too large to enclose in a Stericon holder which has a depth of 4 millimeters. Likewise, total volume in the 250 ml bag, Code 4R5461 (Changed to 4R9952), should not exceed 60 ml. Each cryobag has two female leads ('pigtails') for transferring DMSO-medium and specimen. The plastic tubing adjacent to the bag seals easily in the Sebra sealer. Two sterile pods remain at the top center of the cryobag for use after thawing. Fenwal cryobags (Baxter Health Care Corporation, Deerfield, IL) have been used with exceptional results (<0.06% breakage). A Fenwal blood component infusion set (bcis), Code 4C2223, produces a closed system thereby acting as an added measure against contamination plus, it contains a filter (pore size of 170-210 microns).

Inject contents of 10 cc syringe into conical tube(s). Loosen the cap and pipet 1.1 or 2.1 ml of specimen into the "immix" tube. Dispose of pipet. Using 60 cc syringe, withdraw specimen (the amount should be half the total calculated volume for each cryobag, *e.g.*, 50 ml total volume = 25 ml specimen + 25 ml DMSO-medium)

then close clamp to specimen bag. Remove Luer adapter cover from one cryobag lead and attach bcis coupler. Open clamps on cryobag-bcis and inject specimen.

Pull about 5 ml of air back into syringe and inject it to push through the specimen caught in the tubing. Close all clamps on cryobag-bcis. If additional bags are needed, attach a small syringe to the cryobag lead and attach next cryobag to bcis and repeat procedure. Under hood, use Sebra sealer to seal the cryobag tubing (between clamp and base of Y-shaped tubing) that was used for injecting specimen, pull off and discard. If specimen bag is to be sent for microbiology testing, inject any specimen caught in tubing back into specimen bag, seal specimen bag near base of tubing, pull off tubing, and send bag. Finish labeling cryobags and holders with final volumes. Chill cryobag(s) and "immix" tube.

Under the hood place DMSO-medium (shaken briskly before placing under hood) and n 16G 1.5" needles, where n = the number of cryobags. Loosen cap on DMSO medium. Open syringe and set aside. Attach needle to syringe (be careful to not touch the tapered end of the syringe as it may rest on the sterile tube or bottle of DMSO-medium). For each volume of specimen, withdraw an equal volume of DMSO-medium. Label syringe if more than one syringe of differing volumes will chill together. Chill in refrigerator for 15 minutes. The temperature of the specimen mixed with DMSO-medium must not reach room temperature before commencing 'freeze'.

B. The Cryovials

This step is performed just before starting the freeze program. Cryovials are labeled with the patient's initals, specimen type and unique number, hospital number, and date, and are pre-chilled with the cryobags and holders.

Place 4 to 8 labeled cryovials in a crycrack and the "immix" tube and DMSO-medium in the hood, in 0°C wet ice. Just prior to putting them in the hood, shake the DMSO-medium and vortex the "immix" tube. Under the hood, loosen the caps on

each. Pipet volume of DMSO-medium equal to volume of specimen in "immix" tube, *e.g.*, 2.1 ml, and add to "immix" tube of specimen. Dispose of pipet, tighten cap and vortex mixture. Loosen "immix" tube cap. Pipet specimen, put in cryovial, tighten cap, and submerge in wet ice. Repeat for each cryovial. Remove from hood, put in Styrofoam box and submerge partially in wet ice near freezer. Because of their smaller volume, cryovials of specimen do not have the same eutectic point (Heat of Fusion -- Phase Change) as their cryobag counterparts. Hence, the cryovials are placed in a Styrofoam blood tube box to stagger the rate at which they freeze.

C. The Controlled Rate Freezer Program

This program is written specifically for the Custom Biogenic Systems controlled rate freezer but the principles for freezing stem cells are applicable to any controlled rate freezer. For stem cell cryobag volumes of between 40 and 110 ml, heat of fusion typically occurs between -8°C and -22°C. Ideally, at heat of fusion the specimen heats minimally (between 2°C and 8°C, warming to a temperature that is not warmer than -6°C and returns to the temperature at which heat of fusion occurred within four minutes, certainly no more than 8 minutes, and thereafter. cools at -1°C to -2°C per minute.

Note that the Custom Biogenic Systems (CBS) program 1 includes several "wait" functions serving two functions: 1) to cool the specimen or chamber to a designated temperature and maintain that temperature until the operator advances the program and 2) to enable the operator to advance to the next step by pushing a single button. Those timely seconds saved by pushing one button instead of several are very important at the heat of fusion point. If needed, additional coolant may be added by using the "cool +" button.

TABLE 9

CBS Program 1

Step	Activity	Display
1.1 WAIT	→ + 0° Chamber (Specimen is about + 12 to 14°C)	COOL+
1.2 - 1°/min	→ - 10° Specimen	COOL+
1.3 WAIT	→ - 20° Chamber; heat of fusion ___° Specimen	
1.4 WAIT	→ - 70° Chamber	COOL+
1.5 WAIT	→ - 60° Chamber	COOL+
1.6 +35°/min	→ - 30° Chamber	*WARM*
1.7 -1°/min	→ - 45° Specimen	COOL-
1.8 -10°/min	→ - 90° Specimen.....END	COOL-

Turn on liquid nitrogen. On the computer, advance program to step "1". If using Program "1", then Program and Section show "1.1" in green. Above control status highlighted in green appears "cool", "cool +", and "wait". The chamber chills to 0°C and maintains that temperature in accordance with its wait function.

The DMSO-medium and specimen are mixed immediately before cryopreserving the specimen in order to limit the degree to which the DMSO, which is lethal to cells at room temperature, warms. The mixing should be done as quickly as possible without compromising aseptic technique. All labeling should be done prior to mixing. The controlled rate freezer should be close to the hood where the mixing is done and a refrigerator (4°C) directly beneath the freezer chamber or a few steps away, to prevent as much warming as possible. If the liquid nitrogen storage refrigerator is not located next to the freezer, then a dry shipper or a Styrofoam box containing liquid nitrogen can be used to transport the frozen specimen to the liquid nitrogen refrigerator.

Next to the hood, a plasma extractor should be available, and the Sebra sealer should still be on. Choose a bag of specimen and a syringe of equal volume. Quickly put them under the hood, remove the protective cover from the cryobag lead, remove

the needle from the syringe, and attach the syringe to the bag. Remove from hood the coupled bag-syringe, open clamp, and inject DMSO-medium into the cryobag. Do not detach syringe, mix, and place in plasma extractor. Extract excess air into syringe plus a little specimen. If there is more than 60 cc of air, pat the coupled cryobag-syringe back under the hood, disconnect the syringe, eject some of the air from the syringe, reconnect it and place it in the plasma extractor to continue withdrawing air from the bag. Inject specimen, close clamp. Under hood, seal, pull apart and discard syringe and tubing. Put cryobag in holder. If there is more than one bag, refrigerate and repeat steps starting with selecting a bag a specimen.

D. Cryopreservation

The following steps should be done quickly. The chamber temperature should be 0°C and holding. Open the chamber door and place the ribbon thermocouple on the center of the bag (now referred to as the reference bag). Close the holder carefully so as to not cut the thermocouple. Insert the holder horizontally, ports inward, in a horizontal rack.

Push "scan" on the computer, and on recorder push "start". If there are other holders insert them quickly in the rack. Insert the box of cryovials upright in the chamber and close chamber door. As soon as the chamber returns to 0°C, push "run" once to advance the program to -1°/min (Step 1.2). The specimen temperature should now drop at an appropriate rate and does not need to be monitored until it is about -8°C. Use this time for documentation. Just before and definitely after Step 1.3 engages, watch the recorder pen very carefully in order to detect specimen warming. Be prepared to push "run" as soon as heat of fusion occurs (a sudden rightward pen movement). After heat of fusion occurs, quickly return the specimen temperature to the same temperature at which heat of fusion occurred without incurring too rapid a cooling rate thereafter. If the specimen starts to cool quickly, engage the chamber warming Step 1.6 or open the chamber door, but, if the specimen then cools at less than 1°/minute or plateaus, advance to -1°/min cooling (Step 1.7) and toggle between pausing (no coolant added) and a steady cool rate (Step 1.7). Caution should be

exercised, as any step engaged for too long can cause excessive warming or cooling. After the specimen has returned to the point of heat of fusion, monitor it for about 10 minutes to verify that it cools at not more than -2°/min. The program is reliable hereafter and need not be monitored. It is inadvisable to leave the room, however, in case liquid nitrogen runs out prematurely, electrical outage, *etc.*

The computer will alarm visibly and audibly when finished. Push "run", turn off liquid nitrogen. Put on cryogloves, open the chamber door and put holder(s) except for the reference bag into the shipper or Styrofoam box. Be gentle but quick, and do not drop them. Remove the thermocouple from the reference bag, lift the pen, re-close the holder on the reference bag and put into the shipper or Styrofoam box. Put box of cryovials in shipper or Styrofoam box. If not already done, label frame with patient's last name on left side and date on right and note placement of cryobag(s) in liquid nitrogen refrigerator and position of cryovials in cryovial boxes. Transport holders along with box of cryovials to storage in liquid nitrogen refrigerator. Put holder(s) in frame, hinges facing in, and submerge in liquid nitrogen refrigerator.

If stem cells could follow the ideal freeze curve, they would cool at -1°C/minute until reaching -45°C, thereafter cooling at -10°C/min until reaching -90°C. Heat of fusion, if visible, would be a tiny knoll on the graph, having been successfully anticipated and accounted for automatically by the controlled rate freezer program. However specimens are derived from various sources using different means and do not undergo ficol procedures, vary widely in volume, cell count, and cell composition, thus giving a variable heat of fusion point which requires manual intervention. Optimally, the intervention minimizes the warming of specimen temperature and number of minutes to return to heat of fusion. Flexibility in the program is required and discretion is called upon in order to manually control the specimen's freeze curve through this critical point, heat of fusion. The most difficult aspect is judging whether the specimen temperature is decreasing properly just after heat of fusion has occurred and knowing when to advance to the next step. For

example, if a specimen undergoes heat of fusion at -16°C and warms to -11°C, the rate at which the specimen returns to -16°C can be -5°C/minute or faster without compromising the specimen. However, after returning to the heat of fusion, the rate does matter and should not exceed -2°C/minute. Any manipulations cooling or warming the chamber has a latent effect on the specimen rate after it returns to heat of fusion. The effect those actions have must be anticipated. The cues to a successful freeze manually through heat of fusion are visual.

Cool the chamber the moment heat of fusion is detected. If after initiating extreme cooling of the chamber, the specimen cools immediately, prevent excessive cooling by advancing to a step that cools the chamber less or to a step with one degree per minute cooling of the chamber. If the specimen continues to cool excessively, pause the program or open the chamber door. If the specimen warms after heat of fusion, prevent a plateau by adding coolant through short 'bursts' of cool +. Avoid keeping the chamber more than 20 degrees colder than the specimen for more than two minutes. If the chamber temperature reaches its maximum cold temperature and the specimen starts to warm, even slightly, or seems to plateau, advance to -1°C/min cooling (Step 1.7) and allow the specimen to start cooling until the warming trend is reversed. Or, use 'bursts' of cool +.

As a rule of thumb, the higher heat of fusion occurs, -12°C or higher, the less the specimen warms. In other words, a heat of fusion which occurs at -10°C might warm only 2 degrees to -8°C and return to -10°C in 12 to 60 seconds, leaving little lime for decision-making. If the chamber is over-cooled and the warming step is sorely lacking, open the chamber door to warm quickly, keeping an eye on the sample temperature, not the chamber temperature. For the Linseis graph recorder, each specimen pen marking represents 10 seconds whereas the recording of the chamber - the 'spike'- represents 2 seconds. Hence, five of these 'boxes' equals a minute. Ideally, the specimen temperature will take 5 'boxes' to lower one (or two) degrees.

Toggle between steady cooling (Step 1.7) and warming (Step 1.6 or opening the door), nursing the specimen back to the heat of fusion temperature while also endeavoring to ensure a rate of -1°C (or -2°C) thereafter. The danger in adhering too closely to the formula above (minimizing the warming of specimen temperature and number of minutes to return to heat of fusion) is that the chamber will cool too much or for too long, thus engendering a specimen cooling rate of much greater than -2°C /minute hereafter. Rates of more than 10°C per minute are detrimental to the specimen.

EXAMPLE 7

Selection of CD34+ Cells

The following procedure is used when using the Isolex 300i for the immunomagnetic selection of CD34+ cells. The reagents include one vial of PR34+ Release Agent Dulbecco's Phosphate Buffered Saline (Ca^{++} and Mg^{++} free), Immune Globulin; Intravenous (Human), Human Serum Albumin (25% Solution), Sodium Citrate (4% Solution), and Working Buffer (DPBS; Ca^{++} and Mg^{++} free), supplemented with 1% HSA and 12% Sodium Citrate (v/v). The working buffer should be prepared by mixing 3000 ml of DPBS, 360 ml of sodium citrate and 80 ml of 25% HSA. The buffer may be stored in the refrigerator, but should be warmed to room temperature prior to use. The buffer should be used within 24 hours of preparation.

Prepare a 5% Immune Globulin Intravenous (Human) according to the manufacturer guidelines. Aseptically pull the contents of the Antibody Vial (approx 2.5 ml) into a syringe. Aseptically pull the contents of the PR34+ Stem Cell Releasing Agent Vial (approx 20 ml) into a syringe.

The Dynabeads® should be prepared fresh each day. Remove the contents of one vial of tube for a total of 20 ml. Expose the Dynabeads to the MPC-1 magnet for 2 minutes. Aspirate the supernatant. Remove the magnet and resuspend the beads in

10 ml working buffer. Avoid foaming to minimize bead loss. Transfer the beads to a syringe.

Collect an enriched mononuclear cell product containing up to 4.5×10^{10} cells from mobilized peripheral blood using a peripheral blood stem cell collection. Spike the apheresis product bag with a sampling site coupler and weigh the bag. Cleanse septum with a alcohol wipe, and remove a well-suspended sample with a needle and syringe (2 ml). Determine the nucleated cell count and viability. Save samples for hematology/differential, Immunophenotyping, Tumor Cell Detection (autologous transplants) and microbiology.

Sterile connect a 300 ml plasma transfer set to the collection tubing of a Y-site blood infusion set (with filter) and close the roller clamp on the collection tubing. Sterile connect the 300 ml Transfer Pack filled with working buffer to the blood infusion set and close the roller clamp. Attach the mononuclear cell product to the other input tubing and close the roller clamp. Open the roller clamps on the mononuclear cell product tubing and collection bag tubing, allowing the mononuclear cells to drain into the collection bag. After the cells have drained, close the roller clamp on the collection bag tubing. Open to roller clamp on the buffer bag, allowing the buffer to flow into the product cell bag. Close the roller clamp on the buffer bag and open the clamp on the collection bag allowing the buffer rinse to flow into the collection bag.

Repeat the above steps until approximately 200-250 ml of cell product/buffer have been added to the collection bag. Weigh the collection bag. When ready to install set, cleanse rubber septum on apheresis bag with an alcohol wipe and insert 10 ml of prepared Immune Globulin Intravenous with a needle and syringe.

Turn on the main power switch of the IsolexTM 300i Magnetic Cell Separator located on the back panel of the device. The lights next to the "error" and the "start" keys on the keypad will light momentarily. The identification screen will appear.

This screen is displayed while the instrument is undergoing self test. During the last portion of self test, text indicating the IsolexTM 300i software version number is displayed in the lower right corner of the screen. Without any further intervention by the user, the System Stop Verification display should appear. This is an internal test for the system. At this time the user should press "stop" on the keypad. The System Initialization Preparation display will appear. The operator should follow the instructions to clear the machine and then press "OK." The System Initialization display will indicate the percentage of the System Initialization Tests completed. While the System Initialization screen is displayed, the scale and pressure reference values are obtained and the mechanical parts of the instrument are tested.

Once the tests are finished, the "Select a Procedure" display will appear. Select the Positive Selection application by pressing the box beside "Positive Selection" and then pressing the "OK" box. The "Install Set" display will appear. Remove the tray containing the disposable set from bag. Open the pump door. If possible, hold the tray lengthwise with the chamber next to weight scale arm. Hang the two large waste bags on the holder on the right side of the machine. Install the chamber in the rocker module. Hang both recirculation wash bags on Weight Scale 5. Hang the end product bag on Weight Scale 4. Hang the antibody bag on Weight Scale 3. Hang the release agent bag on Weight Scale 2.

Loosen all of the disposable set parts remaining in the tray and remove the tray. Install the spinning membrane assembly into the spinner module, being sure that after the support arm is in place the spinning device is correctly seated. Lock the upper two clamp manifolds in place. Snap the pump organizer in place. Verify that all tubing in the organizer is seated on the pump rollers or in the grooved surface of the pump module cover. Close the pump door, verifying that the PI tubing and spinning device tubing are not being pinched by the door. Lock the lower three clamp manifolds in place.

Install the secondary magnet pouch on the secondary magnet using the tubing guides. Matching the blue dots, install tubing in Fluid Detectors 1, 2, and securely attach tubing to Pressure Transducers 1 and 2. Matching the blue dot, install tubing in Fluid Detector 3. Install tubing in the rocker tubing guide. Drape the cell source bag and buffer bag tubing such that they do not interfere with the tubing from the bags on the scales. Check to ensure bags are hanging straight on weight scales. Check the disposable set for kinks and pinches.

After the disposable set is properly installed, press "OK" on the "Install Set" screen. The "Install Check" display will appear. The various tests conducted verify proper disposable set installation. P1 and P2 pressures are displayed in the bottom windows on the screen.

Once the installation check is complete, the "Connect Buffer" display will appear. The user should spike port of working buffer bag with disposable set buffer line spike and hang bag on Weight Scale 6, verify that clamps on the working buffer bag are open, and when the working buffer bag is stationary, press "OK." The Prime Set Display will appear. During the Prime Set display, the disposable set is automatically primed using a sequence of steps. The display is updated to reflect the step in progress. The weight scale values are also displayed.

When the Prime Set state is completed, the Add Release Agent screen is displayed. The user should cleanse septum of the release agent bag on Weight Scale 2 with an alcohol wipe, add the contents of the syringe containing the release agent, check that the weight scale bags are hanging straight on the scales, and press "OK." The 'Add Antibody' display will appear. The user should cleanse septum of the antibody bag on Weight Scale 3 with an alcohol wipe, add the contents of the syringe containing the monoclonal antibody, check that the weight scale bags are hanging straight on the scales, and press "OK." The 'Add Beads' display will appear. The user should cleanse septum of the chamber with an alcohol wipe and inject contents of one vial of washed Dynabeads®.

Aim for center of the chamber to avoid deposit of beads on chamber walls, then press "OK." The 'Connect Cell Source Bag' display will appear. The user should spike cell bag with C1 line spike provided on disposable set, hang bag on Weight Scale 1, check that the cell bag tubing is not in the path of the rocker module, and check that the weight scale bags are hanging straight on the scales.

After connecting cell source bag, press OK. At this part, the instrument will automatically begin the selection procedure, running through the following steps: add buffer to reagent; platelet wash; antibody transfer; cells/antibody incubation; antibody wash; transfer cells to chamber; rosetting; chamber cell wash 1-3 release agent transfer cell release incubation; cell release rinse; clear released cells; release agent wash; and transfer cells to and product bag.

After completion of the procedure, the Procedure Complete display will appear. The user should perform the following steps: 1) Close clamp to end product bag (Weight Scale 4); 2) Heat seal the end product bag tubing; 3) Heat seal both pressure transducer tubing; 4) Heat seal buffer bag (Weight Scale 6) tubing; 5) Heat seal primary chamber tubing; 6) Heat seal cell source bag (Weight Scale 1) tubing; 7) Remove end product bag from Weight Scale 4 (ensure end product bag is properly labeled); 8) Remove disposable set from instrument and dispose of in accordance with all applicable regulations regarding biohazardous waste; and 9) Press "OK." When "OK" is selected, the End of Procedure Selection display will appear.

EXAMPLE 8

Seeding Tissue Culture Flasks, Adding Fresh Media to Flasks, and Splitting Cell Cultures

The Fenwal Solution Transfer Pump® is a positive displacement fluid pumping system intended for the accurate transfer of large and small volume laboratory solutions. It employs the Solution Transfer Pump, a Lifecell Transfer Set and Lifecell

Tissue Culture Flasks to provide safe, fast and accurate pumping of a wide variety of fluids.

The system has three principal elements. First is the Solution Transfer Pump, an electromechanical device which pumps opaque and transparent solutions to operator programmed volumes. The pump features gravimetric weighing of pumped solutions to monitor the actual volume of solutions pumped. The pumping unit is designed to perform two types of solution delivery for the laboratory; large volume (red and green pumps) and small volume (orange pump). The red and green pumps are electronically connected to run simultaneously and transfer large volumes of solution. In this Operating Procedure, the red and green pumps will be used solely for the delivery of fresh culture media to the culture flasks. The orange pump operates individually and transfers small volumes of solution more accurately. The orange pump will be used to transfer existing cell cultures from a flask to one or more flasks or in some instances it is not utilized at all. Second is the Lifecell Transfer Set, a sterile, multiple use fluid transfer set, and third is the Lifecell Tissue Culture Flask, a sterile, single use container.

In certain embodiments, the cell cultures being handled and processed are of cells collected from HIV seropositive individuals. During tissue culture flask seeding, the addition of fresh media to cell culture flasks and the splitting of cell cultures, an efficient and safe method that safeguards the sterility of the cultures is essential. In addition, for the protection of the processing personnel, developing a method that provides a closed system, reducing the need for unnecessary exposure to biohazardous fluids and the use of sharps is of high priority.

The supplies and equipment include the Fenwal Solution Transfer Pump (Baxter Cat. # 4R4345), the Fenwal Lifecell Transfer Set (Baxter Cat # 4C2474), the Fenwal Lifecell Adapter Set (Baxter Cat # 4C2476), a Laminar Flow Hood, and a Sebra Dielectric Heat Sealer. These are standard supplies and equipment used for all

the procedures described below. Any additional supplies and equipment required for specific tasks will be listed under each procedure individually.

A. Initial Startup

First, the transfer pump is initialized. The cable connections from control module to pump module and the power cord connection to pump module should be connected securely. Turn on the pump module rear power switch. The membrane panel power on indicator should illuminate and remain on. The power-up self-test should occur as specified in the Operator's Manual. The self-test ends with all displays showing a right justified zero with the "g" indicator illuminated. If problems occur, do not use the pump and refer to Section 6 of the Operator's Manual (Troubleshooting Guide). Allow the pump to warm-up for approximately five minutes before proceeding. Perform Load Cell Performance Check. Refer to Section 7.2 of the Operator's Manual for the procedure.

Next, the transfer and adapter sets are set up. Remove the Transfer Set from the pouch. Ensure that the Transfer Set Junction Cover is securely closed over the Junction port opening. Pre stretch each of the silicone segments before starting to place the Transfer Set on the Transfer Pump. Grasp the segment by the end connectors and slowly stretch approximately two inches and relax the segment. Load Transfer Set onto the Pump Module as follows: a) Open safety door on pump module; b) Insert transfer set junction into junction holder on pump module; c) Ensure the color coding of the pump segments matches the color coding of the pump heads. Starting at the right (green) pump station, insert the white connector into the white outlet tubing guide (right side guide of the pump station) so that the connector rests on the top surface of the guide; d) Route pump tubing around the rollers of the pump rotor, stretching tubing outward; e) Position tubing into tubing guide slot without twisting and allow green connector to retract into green tubing guide; f) Rotate pump rotor counterclockwise at least one turn to seat tubing around rotor; g) Repeat Steps (c) through (f) for the center (orange) and left (red) pump stations. All tubing segments must be installed in pump rotors for proper instrument operation; and h)

Close the safety door and ensure that the transfer set tubing is not twisted, kinked or clamped. Remove the Lifecell Adapter Set from the pouch. Connect the red and green pump segment couplers to the end of the Adapter Set that has two ports. The single port on the Adapter Set will be used to connect the cell culture media container.

Next, the solution transfer pump is programmed. Press the volume control pad for the display to be programmed. The audible signal sounds once and the display flashes. Using the numeric keyboard, enter the volume for the corresponding solution. As data is entered, each new digit shifts sequentially from right to left in the display. Ensure that the volumes entered for each pump correspond to the solutions and amounts that are connected to each pump station. It is recommended that one half of the total amount of fresh Culture Media to be transferred be entered on each of the red and green pumps. This allows for the extension tube on the final container to be both primed and rinsed with Culture Media. Press specific gravity control pad for the display to be programmed. The audible signal sounds once and the display flashes. Using the numeric keyboard, enter the specific gravity for the corresponding solution. As data is entered, each new digit shifts sequentially from right to left in the display. A specific gravity of 1.00 may be used for most cell culture solutions.

B. Seeding Of Tissue Culture Flasks

The Lifecell Tissue Culture Flask of the desired size, the Cell Culture Media Container, the Plasma Transfer Set (Baxter Cat # 4C2243), the Terumo SCD312 Device/Welding wafers and the container with the cells to be seeded should be gathered inside the laminar flow hood. Using the SCD312, connect the Plasma Transfer Set to the tubing already attached to the Cell Culture Media Container. Ensure that the roller clamp on this line is closed. Connect the Media Container to the Adapter Set already installed on the Transfer Pump. Use the coupler on the Plasma Transfer Set and the single port of the Adapter Set. Open the roller clamp to the media container. Carefully uncap the junction as to prevent contamination of the junction port. Connect the Lifecell Flask to the transfer set junction using the Junction Coupler on the flask. Close all the roller clamps on the flask except the one

to the junction coupler. Hang the Lifecell Flask from the final container hook on the Load Cell. Ensure that the flask hangs freely with no restrictions that may alter the operation of the Load Cell.

Connect the cell container to the orange pump tubing coupler and hang from one of the container hooks of the pump module. The Solution Transfer Pump is designed to accommodate solutions in collapsible containers. If the cells to be seeded are in a glass bottle or other noncollapsible container, the container must be vented to permit the solution to flow properly. Enter the volume of Culture Media to be delivered to the final container (Lifecell Flask connected to the pump junction). This volume must be divided between the red and green pumps. For example, if 500 ml of media are to be transferred into the final container, program the red pump for 250 ml and the green pump for 250 ml. Enter the volume of the cell suspension to be seeded by programming the orange pump. For example, if the total volume of the cell suspension is 200 ml, program the orange pump to deliver 200 ml if you want all the cell suspension to be transferred to the final container. Enter the specific gravity of the solutions being transferred on each pump. A specific gravity of 1.00 is normally programmed for each pump unless otherwise directed by the Head, Clinical Cell Production Facility.

Before proceeding any further, the following conditions should exist. The final container should be properly connected to the transfer set junction, the junction coupler clamp of the final container should be open while all the others are closed, the culture media container clamp should be open, there should be no kinking or clamping of the transfer set tubing or final container extension tube, and the total programmed volume should not exceed the volume capacity of the final container.

Press the start pad on the control module to begin the pumping cycle. All volume displays show four zeros and the ml indicator illuminates. As the transfer from each pump station occurs, the volume displays show actual volume transferred with leading zeros for the respective station. In addition, the cumulative transferred

volume in ml is shown in the total delivered display. The pumping cycle is as follows: a) The red and green pumps simultaneously transfer the volume initially programmed into the red pump; b) The orange pump individually transfers the volume initially programmed into the orange pump; and c) The red and green pumps simultaneously transfer the volume initially programmed into the green pump.

If the pumping cycle needs to be stopped, press the stop/mute pad. The pumping cycle underway is aborted and cannot be restarted. Remove the final container and start a new cycle with a new final container. If the pumping cycle needs to be stopped for any reason, but aborting the cycle underway is not desired, simply open the pump module cover. This will cause the pumping cycle to be halted temporarily without the need for reprogramming the pumping cycle. To restart the pumping cycle, close the pump module cover and press the start pad. The pumping cycle previously underway will resume at the point where it was halted. At the completion of the pumping cycle, the green complete LED indicator illuminates and an audible signal sounds and pumping action automatically stops. The volume displayed in the total delivered display should equal the sum of the volumes programmed into each pump. For example, using the volumes used as examples on Steps 7 and 8, the total delivered display should read 700 ml. A positive or negative deviation of one to two ml in the total delivered display is acceptable.

Without disconnecting the final container connector from the junction, remove the final container from the hook on the Load Cell and place it next to the junction. Using the Sebra Heat Sealer, seal the junction coupler tubing of the final container three times as close to the junction coupler as possible and in the direction of the container. Close the roller clamp on the junction coupler tubing. Separate the final container from the junction coupler by detaching the tubing from the heat seal closest to the junction coupler. This way, two and a half heat seals remain on the tubing attached to the final container and a half of a heat seal remains with the junction coupler. Label the final container as appropriate and use the volume that was displayed in the total delivered display as the volume of the final container. Store the

cell culture container (Lifecell Flask/final container) in a humidified CO_2 incubator at 37°C. Heat seal the tubing connected to the cell culture media container twice leaving enough tubing length for future SCD312 connections. Detach the media container from the Solution Transfer Set and store in its box inside a 4°C refrigerator. Remove all the disposables from the Solution Transfer Pump and dispose of them in accordance to the Infection Control Manual. Disinfect the Transfer Pump with a 50% solution of Sodium Hypochlorite and water. Clean the Laminar Flow Hood with 70% Ethanol solution.

C. Adding Media To An Existing Cell Culture Flask

Refer to the previous sections for preliminary steps before adding media to an existing cell culture flask. Perform the Transfer Pump Startup and the Transfer Pump Setup, and program as specified in Section A above. Program the Transfer Pump as specified in Section A above, except that no volume will be programmed into the orange pump. In this procedure, fresh media is being added to an existing cell culture flask and only the red and green pumps are used to transfer the solution.

Connect a Plasma Transfer Set to the media container as specified in Section B above. Connect the media container to the Adapter set as specified in Section B above. Gather the Terumo SCD312 Device, a Sterile junction coupler from a Lifecell Flask, and a Lifecell Flask containing the cell culture inside the Laminar Flow Hood. Using the SCD312, connect the junction coupler to one of the tubing extensions attached to the cell culture container. Connect the junction coupler on the Culture Container to the Junction port of the Transfer Set as described in Section B above. Hang the cell culture container from the hook on the Load Cell as specified in Section B above.

Program the red and green pumps with the volume desired, and program the specific gravity, as specified in Section B above. Perform the checks specified in Section B above before proceeding. Press the start pad to start the pumping cycle. Refer to Section B above for details and guidance. The following exceptions apply: a)

The orange pump is not programmed, therefore any reference made to the orange pump in the steps listed above does not apply to this specific procedure; and b) The volume displayed in the total delivered display is the sum of the volumes programmed into the red and green pumps.

Label the cell culture container as appropriate. Ensure that the correct volume is recorded on the culture label. The volume in the culture container is now the sum of the initial culture volume plus the volume displayed in the total delivered display. For example, if the volume of the cell culture container before fresh media was added was 1000 ml and the total delivered display shows a volume of 500 ml (this volume should be equal to the sum of the programmed volumes of the red and green pumps +/- 2 ml), the cell culture container now holds 1500 ml. Store the cell culture and seal off the cell culture media container as specified in Section B above. Remove all the disposables from the Solution Transfer Pump and dispose of them in accordance to the Infection Control Manual. Disinfect the Transfer Pump with a 50% solution of Sodium Hypochlorite and water. Clean the Laminar Flow Hood with 70% Ethanol solution.

D. Splitting Cell Cultures (1:2 Split)

Determine the ratio of cell culture to fresh media necessary to attain the cell concentration desired prior to starting setup. Refer to the previous sections for preliminary steps before adding media to an existing cell culture flask. Perform Transfer Pump Startup and Perform Transfer Pump Setup as specified in Section A above. Program the Transfer Pump as follows (refer to Section A above): a) Red Pump - half the total volume of fresh media desired in the final container; b) Green Pump - half the total volume of fresh media desired in the final container; and c) Orange Pump - half the total volume in the cell culture container to be split. For example, the cell culture container to be split has a total volume of 1500 ml. The culture is to be split into two new Lifecell Flasks with a total volume of 1000 ml each. 750 ml of the existing cell culture will be transferred into each individual Lifecell Flask. The total volume of fresh media required is 500 ml which will be equally

divided (250 ml each) between the two new Lifecell Flasks. Therefore, program the pumps as follows: Red Pump (media) - 125 ml / Green Pump (media) - 125 ml / Orange Pump (cell culture) - 750 ml. The total volume transferred to each new flask will be 1000 ml.

Connect a Plasma Transfer Set to the media container, and connect the media container to the Adapter Set as specified in Section B above. Gather the Terumo SCD312 Device/Wafers, a Sterile Junction Coupler from a Lifecell Flask (1), Lifecell Flasks (2) - size as required and a "Y" juncture from a 600 ml Transfer Pack with 8 Couplers (1) (Part of Baxter Cat # 4R2027) inside the Laminar Flow Hood. Using the SCD312, connect the junction coupler to the single end of the "Y" juncture. Using the Sebra Heat Sealer, seal off the junction couplers of each of the new Lifecell Flasks. Detach the couplers and store in a plastic bag for future use. Ensure that the roller clamps on the junction coupler tubing remain attached to the flask. Using the SCD312, connect the tube extension of the Lifecell Flask that formerly had the junction coupler at the end to one of the available legs of the "Y" juncture. Close all the roller clamps. Using the SCD312, connect the tube extension of the other Lifecell Flask to one of the available legs of the "Y" juncture and close the roller clamps.

Connect the junction coupler attached to the two Lifecell Flasks to the junction port of the Transfer Set as specified in Section B above. Hang one of the Lifecell Flasks on the hook of the Load Cell as specified in Section B above. Open the roller clamp on the tubing line that connects to the "Y" juncture. The red, green and orange pumps should already be programmed as specified above in this Section. If not, program the pumps now. Program the specific gravity for each solution as specified in Section B above. A specific gravity of 1.00 should usually be entered for each solution.

Before proceeding any further, the following conditions should exist. The final container should be properly connected to the transfer set junction, the junction coupler clamp of the final container should be open while all the others are closed, the

culture media container clamp should be open, there should be no kinking or clamping of the transfer set tubing or final container extension tube, and the total programmed volume should not exceed the volume capacity of the final container.

Remove the cell culture container to be split from the incubator and place inside the Laminar Flow Hood. Resuspend the cells by shaking the container. Connect the orange pump tubing coupler to the culture container using the port farthest from the tubing extensions attached to the container. Hang the cell culture container from one of the container hooks of the pump module. Press the start pad to start the pumping cycle. Refer to Section B above for details and guidance. At the completion of the pumping cycle, the green complete LED indicator illuminates and an audible signal sounds and pumping action automatically stops. The volume displayed in the total delivered display should equal the sum of the volumes programmed into each pump. A positive or negative deviation of one to two ml in the total delivered display is acceptable.

Close the roller clamp on the tube extension of the flask hanging from the hook of the Load Cell. Remove the flask from the hook and place next to the junction. Hang the other Lifecell Flask from the hook of the Load Cell and open the roller clamp on the extension tube connected to the "Y" juncture. Ensure that the tubing lines are not kinked or tangled in any way that may affect the flow of fluid or the operation of the Load Cell. Press the start pad to start the pumping cycle. Refer to Section B above for details and guidance. At the completion of the pumping cycle, the green complete LED indicator illuminates and an audible signal sounds and pumping action automatically stops. The volume displayed in the total delivered display should equal the sum of the volumes programmed into each pump. A positive or negative deviation of one to two ml in the total delivered display is acceptable. Close the roller clamp on the tube extension of the flask hanging from the hook of the Load Cell. Remove the flask from the hook and place next to the junction. The cell culture split is now complete.

Using the Sebra Heat Sealer, seal the Lifecell Flasks' tubing lines connected to the "Y" juncture three times. Ensure that the roller clamps remain with the flasks. Disconnect the flasks from the "Y" juncture by separating the heat seal closest to the "Y" juncture. This way the flasks have two and a half seals in their lines and a half seal remains on each leg of the "Y" juncture. Label each new culture container as appropriate and store as specified in Section B above. Seal off the cell culture media container and store it as specified in Section B above. Remove all the disposables from the Solution Transfer Pump and dispose of them in accordance to the Infection Control Manual. Disinfect the Transfer Pump with a 50% solution of Sodium Hypochlorite and water. Clean the Laminar Flow Hood with 70% Ethanol solution.

E. Splitting Cell Cultures (2:4 Split)

The 2:4 split of cell cultures is identical to the 1:2 split except for the following. Instead of two Lifecell Flasks, four are required, a double "Y" juncture from a 600 ml Transfer Pack with 8 Couplers is required instead of a single "Y" juncture (Baxter Cat # 4R2027), and a 5-Prong Manifold is added to the required supplies (Baxter Cat # 5C4446). The junction couplers of the Lifecell Flasks are still sealed off but in this procedure it is done four times since there are four flasks. The SCD312 is used to connect the flasks to the double "Y" juncture. The only difference is that a double "Y" juncture has four available legs which correspond with the number of Lifecell Flasks used in this procedure. This repeated for each flask.

Since this is a 2:4 split, indicating that two existing cell cultures are being split into four new Lifecell Flasks, two cell culture containers are removed from the incubator. The 5-Prong Manifold is used to convert the orange pump coupler from single to double - this eliminates the need to disconnect a culture container from the orange coupler to connect another. To do this, using the Sebra Heat Sealer, seal off three of the prongs on the 5-Prong Manifold. The 5-Prong Manifold now has only two couplers at one end which will be used to connect the cell culture containers to the orange pump. On the other end of the manifold there is a coupler port. Connect the orange pump tubing coupler to this port of the manifold. Ensure that the clamps

on the two remaining prongs of the manifold are closed. Using the couplers at the end of the prongs, connect the two culture containers to be split to the orange pump and hang the containers from the hooks on the pump module.

Open the clamp on one of the culture containers and press the start pad to start the pumping cycle. The cell culture on the container now open will be transferred in equal amounts (half the total volume) into two of the four Lifecell Flasks connected to the Transfer Set junction. Once this culture container is empty, close its clamp and open the clamp to the other culture container. Press the start pad to start the pumping cycle. Refer to Section B above for details and guidance. At the completion of the pumping cycle, the green complete LED indicator illuminates and an audible signal sounds and pumping action automatically stops. The volume displayed in the total delivered display should equal the sum of the volumes programmed into each pump. A positive or negative deviation of one to two ml in the total delivered display is acceptable. Close the roller clamp on the tube extension of the flask hanging from the hook of the Load Cell. Remove the flask from the hook and place next to the junction. Hang the other Lifecell Flask from the hook of the Load Cell and open the roller clamp on the extension tube connected to the "Y" juncture. Ensure that the tubing lines are not kinked or tangled in any way that may affect the flow of fluid or the operation of the Load Cell. This is repeated for each Lifecell Flask.

Using the Sebra Heat Sealer, seal the Lifecell Flasks' tubing lines connected to the "Y" juncture three times. Ensure that the roller clamps remain with the flasks. Disconnect the flasks from the "Y" juncture by separating the heat seal closest to the "Y" juncture. This way the flasks have two and a half seals in their lines and a half seal remains on each leg of the "Y" juncture. This is repeated for each Lifecell Flask.

In most instances, the volume on each cell culture container to be split will be the same. This simplifies the splitting process since once the transfer of cell culture and fresh media into a Lifecell Flask connected to the Transfer Set junction is complete, all that is required is that the roller clamp to that flask be closed, the flask

removed from the Load Cell hook and replaced with an empty flask whose clamp is opened and the start pad pressed to start a new pumping cycle. In cases that the volumes of the two cell cultures being split are different, reprogramming of all the pumps would be necessary once the first culture container has been split. This is determined prior to starting the splitting process but does not require any other alteration in the procedure besides the reprogramming of the pumps between cultures.

Any fluid leakage or disposable set disconnection constitutes a break in sterility and the set must be discarded and replaced. The cell cultures being processed should be considered contaminated. Should the break in sterility occur during the actual solution transfer process, turn the Transfer Pump off, close all the clamps on the Transfer Set and all related tubing lines and contain the spill of fluid per standard guidelines. Although this procedure is performed inside a Laminar Flow Hood it is recommended that all personnel in the processing room leave the premises immediately to prevent exposure to any aerosol created by the break on the tubing lines.

EXAMPLE 9

Ex Vivo Expansion of $CD28^+/CD4^+$ T Lymphocytes via Bead-Immobilized Anti-CD3 Antibody (OKT3) plus Anti-CD28 Antibody(9.3)

The two-signal model of lymphocyte activation states that lymphocytes require the delivery of both an antigen-specific signal as well as a simultaneous costimulatory signal. In the absence of a costimulatory signal, interaction of the T cell receptor with the antigen-MHC complex may cause T cell clonal anergy or deletion. Data from many laboratories indicates that CD28 can provide an important costimulatory signal.

The proliferative potential of T cells expanded *in vitro* is a major consideration for adoptive immunotherapy. Antigen-specific and polyclonal $CD8^+$ T cells have been successfully expanded *in vitro* by the addition of IL-2 or anti-CD3 Ab + IL-2.

However, mixed populations of $CD4^+$ and $CD8^+$ T cells stimulated in this manner will eventually result in a population that is all or mostly $CD8^+$. Further, the long-term growth of $CD4^+$ T cells has necessitated the addition of exogenous lymphokines and allogeneic feeder cells, which precludes a large-scale expansion of $CD4^+$ T cells for the treatment of disease.

The procedure described here is a method for expanding purified $CD4^+$ T cells independent of exogenous cytokines or feeder cells using anti-CD3 antibody (Ab) plus anti-CD28 Ab conjugated to magnetic beads. Autocrine growth in normal donors is maintained for a 4-6 $\log_{10}$ fold expansion, and in HIV^+ donors for a 3-5 $\log_{10}$ fold expansion. These cells remain polyclonal and >97% $CD4^+$. In some patients, the addition of IL-2 enhances growth of lymphocytes, and thus this is an optional step in certain embodiments. Activated cells secrete predominantly cytokines associated with T helper type I function.

A Coulter Multisizer IIe equipped with Channelyzer interfaced PC with AccuComp software, a Magnetic Particle Concentrator (MPC-1; P & S Biochemicals, Gaithersburg, MD); Magnetic Separator for 15-50 mm tubes (Collaborative Biomedical Products, Bedford, MA); Magnetic Separator for flasks (Collaborative Biomedical Products, Bedford, MA); and a Rare Earth Cobalt Magnet, (Edmund Scientific Co., Barrington; NJ) were used.

Reagents used included Anti-CD3 (OKT3) plus anti-CD28 (9.3) coated Dynal M-450 Tosylactivated beads; X-VIVO 15 with 5% autologous human serum (BioWhittaker Walkersville, MD); L-glutamine (BioWhittaker Walkersville, MD); and Recombinant IL-2 (Chiron Corporation, Emeryville, CA).

The primary stimulation is referred to as S1, the first restimulation is referred to as S2, the second restimulation is referred to as S3, the third restimulation is referred to as S4, *etc.* The day of stimulation is referred to as D0, 24 hr after

stimulation is referred to as D1, 48 hr after stimulation is referred to as D2, *etc*. For example: S4D3 = 72 hr after the fourth stimulation (third restimulation).

A. Counting Cells Using a Coulter Multisizer IIe

Electronic cell enumeration and volume determinations are more accurate and have more precision than hemocytometer-based counting protocols, and are therefore a preferred method. The operation of the counter is based on the principle that particles (cells suspended in an electrolyte) passing an aperture through which electric current is flowing alter the electrical resistance of the electrolyte and give rise to changes in the current flow and voltage. The magnitude of these changes is directly proportional to the particle size and can be electronically converted to a particle count. Up to 500 particles are individually counted and sized per second, and particle size is determined independently of shape or orientation in solution. In most instruments a lower threshold control is available to select minimum particle size so as to eliminate counting of particles too small to be of interest. The electronic counter utilizes side scatter to distinguish live from dead cells. The operation of the electronic counter is fully explained in the manufacturer's manual.

The sampling stand should be equipped with a 70 micron "long-bore" orifice tube for more accurate counting and sizing. This long-bore aperture predisposes to clogging of the orifice but enhances size discrimination. This ensures that only live cells are counted by eliminating debris. For counting lymphocytes, especially after beads have been added, the cells should be well dispersed to break up any clumps. The lower gate for sizing should be set at 100 fl, and sample volume is set for 0.5 ml. For sizing stimulated cells, set the sizing (analysis) gate at 200 fl and maintain this gate throughout the culture period. This ensures that beads (4.5 micron diameter) are not counted and are electronically gated out.

To count cells, add 19.96 ml of PBS or Coulter diluent to a sampling vial. Add 160:1 of resuspended cells to the vial and count twice. The value next to "coincidence corrected count" is recorded and the number is divided by 8 (2× for two

counts and 4× for dilution factor) to yield the concentration of cells per ml such that #### is #.### × 10^6 cells per ml. A background count of PBS only should read 100 or less. Experience has shown that CD3+28 T cells are best restimulated at 350-400 fl. Clean and disinfect the apparatus using a 10% bleach solution when completed.

B. Cell Culture

On Day 0, add CD34-fraction cells to X-VIVO 15® with 5% autologous human serum at a concentration of 1 × 10^6 per ml. The culture may be started in a 300 ml Baxter Lifecell Flask if the starting amount of cells is at least 50 × 10^6 cells. For details on the use of Baxter Lifecell® Flask and Solution Transfer Pump to culture cells, see Example 8. Wash appropriate amount of tosylactivated beads coated with anti-CD3 plus anti-CD28 Ab (BB IND 6675) 3× with X-VIVO 15® and add to cells, maintaining a concentration of 1 × 10^6 cells per ml.

On Day 3, detach the cells from the antibody-coated beads by shaking/agitation of the bag until the clumps of cells have been dispersed. Count the cells and add fresh media so that the cells are maintained at a concentration of 0.75-2 × 10^6 per ml. The culture should not be diluted more than 1:1 with fresh medium. On Day 4,5,6 *etc.*, detach the cells from the antibody-coated beads by shaking/agitation the bag until the clumps of cells have been dispersed. Maintain the cells as described for Day 3. Monitor cell size as described below in order to assess the correct time for restimulation of the culture.

Standard cell culture technique uses a microscope with a practiced eye to determine the optimal time of culture restimulation. In this protocol cell volume is used to determine the time of cell restimulation. The use of an electronic cell volume determination can reliably discriminate activated from resting cells, and is quicker and easier than counting cells using a hemacytometer. However, unless properly calibrated, electronic cell volume determinations are subject to error due to the presence of cell clumps or beads. In addition, electronic counting requires precise resetting of the instrument for cell populations of different sizes, possibly leading to

inaccurate counts for heterogeneous cell populations and necessitating separate settings for counting of resting-and activated cells. Thus, cell populations derived from tissues that may contain significant numbers of dead cells and cell clumps are difficult to size with an electronic counter. Overall, the electronic cell counter is best reserved for repetitive and rapid sizing of peripheral blood cells in suspension culture.

Resting T cells have a mean volume of ~170 fl, with only a small deviation from the mean (less than 40 fl). Resting T cells from HIV^+ donors have a slightly larger volume of ~200 fl. The activated T cells will reach a maximum mean volume of 780-900 fl by Day 5-8 of stimulation. Also, the deviation from the mean will be larger (250-350 fl). Around Day 7-10, the mean cell volume and the deviation from the mean will begin to fall. Eventually the cells will return to a resting T cell volume. Restimulate the culture when the mean cell volume falls below 400 fl (usually between S1D12 and S1D20). The cells should not be allowed return all the way to a resting cell volume (this will be too late and the cells die). Analyze cell sizing data using Accucomp Software (Coulter). Cell size histograms are generated by the software. Data is stored digitally on backup disks. Different donors will vary as far as the time of first restimulation. The more the culture is restimulated, the shorter the time interval between restimulations.

C. IL-2 Addition

CD28-stimulated cells from normal blood donors do not require addition of exogenous IL-2 to the medium for cell growth. However, cells from some donors may have deficient IL-2 secretion, and in these cases, optimal cell growth is achieved by addition of rIL-2. rIL-2 (Chiron) is added from single use vials, 22×10^6 IU/vial. Add sufficient IL-2 to bring the culture medium to 100 IU/ml.

On S1D11 (Day -2 Reinfusion) an aliquot of each culture is withdrawn, pooled and sent for bacterial culture and sensitivity testing, fungal culture and mycoplasma culture. On S1D11 (Day -2 Reinfusion) an aliquot of each culture is tested for endotoxin using the QCL-1000 chromogenic LAL assay (BioWhittaker, Inc.) and a

SpectraMax 340 microplate reader according to the manufacturers directions. Each test is run in triplicate and is quantitated using a certified endotoxin standard (BioWhittaker, Inc.). On S1D11 (Day -2 Reinfusion) an aliquot of each culture is withdrawn, pooled and tested for mycoplasma using the ATCC Mycoplasma Detection Kit. The PCR reactions are run on either a Perkin-Elmer 9600 or Idaho Technology thermal cycler according to the manufacturer's instructions. Each test is run in triplicate and is validated against two positive standards (ATCC).

EXAMPLE 10

Removal Of Magnetic Beads From Cell Culture Media

In certain aspects of the present invention, the magnetic beads may be removed from cell culture media using the Baxter Fenwal MaxSep® Magnetic Cell Separation System. The Baxter Fenwal MaxSep® Magnetic Cell Separation System consists of the MaxSep Magnetic Cell Separator and the MaxSep Magnetic Cell Separator Disposable Set. The larger Primary Magnet is a strong permanent magnet constructed of Neodymium-Iron-Boron Bars and engineered to provide optimal separation characteristics. It is used to attract the microbeads to the magnet surface allowing the remaining cells and suspension to flow out of the Primary Container. The Secondary Magnet is designed to capture microbeads which may escape the Primary Magnet. The residual cell/fluid mixture is collected in a container (Cell Recovery Container) and then further processed on the Baxter Fenwal Cell Harvester if desired.

The MaxSep System incorporates a peristaltic pump with a flow rate range of 1-30 ml/min. On average, approximately twelve liters of cell suspension are processed. In some procedures, the pump may be bypassed to allow processing of the cell suspension at a faster flow rate. This reduces the total processing time and the time the cells are maintained at room temperature out of the incubator which could affect cell viability. In addition to the pump being bypassed, other modifications are made on the MaxSep Disposable Set to both accelerate the separation process and to

create a semi-closed system. These modifications ensure the sterility of the cell suspension and add protection to the processing staff.

During cell culture, antibody-coated paramagnetic microbeads are used to stimulate cell growth and expansion. Once the cell expansion goal is achieved, it is necessary to efficiently remove the microbeads prior to reinfusion into the patient-donor to prevent or reduce the possibility of undesirable side effects which may be caused by the infusion of these antibody-coated microbeads. The magnetic cell separation procedure is performed the day of scheduled cell reinfusion and prior to cell harvesting or as desired.

The following equipment and supplies were used: a Baxter Fenwal MaxSep Magnetic Cell Separation System (1); Terumo SCD312 Sterile Tubing Welder (1)/ Welding Wafers (as needed); Sebra Dielectric Heat Tube Sealer - Model 2100 (1); IV Pole (1); MaxSep Disposable Set (1) (Baxter Cat # 4R5401); 600 ml Transfer Pack with eight Couplers (2) (Baxter Cat # 4R2027); 1000 ml Lifecell Flask (1) (Baxter Cat # 4R2111); 2000 ml Transfer Pack (8) (Baxter Cat # 4R2041); 1000 ml bag Plasmalyte-A (1) (Baxter Cat # 2B2544) - priming solution; Cell Culture Media contained in 3-liter Lifecell Flasks (8); "Y" Juncture from Plasmacell-C Disposable Set (1) (Baxter Cat # 4R2252); Laminar Flow Hood (1); and a Plasma Extractor (1).

A. Modification of MaxSep Disposable Set

The following procedures should be performed before the cell culture flasks are entered and lines are exposed to fluids. The standard operating procedure for the Terumo SCD312 is followed to maintain system sterility.

Obtain cell suspension samples from each flask to be processed. Determine total cell count and cell viability as per protocol guidelines. Prepare the MaxSep Set as follows: 1) Detach a "Y" juncture from the Plasmacell-C Disposable Set by heat sealing the tubing approximately 3 inches away from the "Y" on each leg using the Sebra Heat Sealer. Carefully remove from the rest of the set and save. Store unused

Plasmacell-C Set in a sealed plastic bag for future use; 2) Using the Sebra Heat Sealer, seal off the Secondary Chamber Line, the Priming Line and the Cell Recovery Line from the unmodified MaxSep Disposable Set. Seal the Secondary Chamber Line and the Priming Line as close to the "Y" connection as possible. The Cell Recovery Line is sealed off approximately 1 inch away from the Recovery Line Bushing eliminating the Pump Segment altogether. Carefully detach these tubing lines from the "Y" and save; and 3) Using the SCD312, weld the single leg below the "Y" juncture from the Plasmacell-C Disposable Set to the Cell Recovery Line - this line will become the Reservoir Bag Line. Weld one of the legs above the "Y" to the Priming Line - this line is the Priming Solution Container Line. The leg that remains is welded to the Secondary Chamber Line. Ensure that all clamps are closed and remain part of the setup.

Prepare the Outlet Set as follows. Individually connect the single tubing leads of the eight 2000 ml Transfer Packs to the eight tubing leads of a 600 ml Transfer Pack with eight Couplers Set using the SCD312. Close all roller clamps after connection. The 600 ml Transfer Pack becomes the Reservoir Bag and the 2 liter Transfer Packs become the Cell Recovery Containers. Discard the spike connectors.

Prepare the Inlet Set as follows. First, using the SCD312, weld the pre-attached lead on the 1000 ml Lifecell Flask above the "Y" juncture to the single tubing lead of the other 600 ml Transfer Pack with eight Couplers Set. This line becomes the Cell Suspension Line. The 1000 ml Lifecell Flask is the Primary Separation Container. Discard the 600 ml Transfer Pack. Ensure that the roller clamp is closed and remains part of the new set up. Second, the eight couplers attached to the Primary Separation Container will eventually be connected to each of the 3000 ml Lifecell Flasks containing the Cell Culture Suspension using the spike ports. For now, ensure that all the roller clamps are closed.

Connect the MaxSep, Outlet and Inlet Sets as follows. Insert the Primary Line Coupler of the MaxSep Disposable Set into the spike port farthest from the

pre-attached lead (Cell Suspension Line) on the 1000 ml Lifecell Flask that will be used as the Primary Separation Container. Connect the Reservoir Bag Line to the spike port farthest from the lead coming out of the 600 ml Transfer Pack (Reservoir Bag) with eight couplers (Cell Recovery Containers). Close all clamps. The single line that exits the Reservoir Bag toward the Recovery Containers becomes the Cell Recovery Line. Connect the Priming Line to the Priming Solution Container (Plasmalyte-A) using the spike port. Ensure that the Priming Line Clamp is closed before connection. Hang Priming Solution Container vertically from a Ring Clamp Stand located inside the Laminar Flow Hood. Once all the connections are completed and ensuring that all clamps on the Disposable Set are closed, proceed with the Priming Procedure described next.

B. Priming Procedure

It is very important to remove all air bubbles from the secondary separation chamber and primary line. Microbeads will strongly adhere to bubbles and can be carried downstream despite the presence of a strong magnet.

Open the Priming Line Clamp and the Reservoir Bag Line Clamp. Do not open the eight clamps (Recovery Containers Clamps) to the 2000 ml Cell Recovery Containers. Allow the Priming Solution to flow into the Reservoir Bag ensuring that all air is cleared from the tubing. Once approximately 200 ml have flowed into the Reservoir Bag, close the Priming Line Clamp and the Reservoir Bag Line Clamp. Air in the Reservoir Bag will be expressed toward the Cell Recovery Containers. Roll up the Reservoir Bag and ensure that the air in the bag is at the top (end of bag where spike ports are located) or place Reservoir Bag in a Plasma Extractor. Open the Cell Recovery Line Clamp and one of the Cell Recovery Container Clamps. Remove the air in the Reservoir Bag by squeezing it or using the Plasma Extractor. Once all the air has been removed from the Reservoir Bag, continue squeezing the bag and allow Priming Solution to flow into the Recovery Container to clear all the air in the line. Close the Recovery Container Clamp.

Continue to apply gentle pressure on the Reservoir Bag. Open the clamp of the next Recovery Container. Clear all air from the line by flushing with the Priming Solution in the Reservoir Bag. Close the Recovery Container Clamp and continue to maintain pressure on the Reservoir Bag. Repeat these steps on the rest of the Recovery Containers until all the air in the tubing is cleared. Close the Reservoir Bag Clamp. Remove Reservoir Bag from Plasma Extractor if applicable. Fill the Secondary Separation Chamber by opening the Secondary Chamber Line Clamp and the Priming Line Clamp. Hold the Secondary Chamber upright, so that all the air is forced toward the Primary Line Coupler. When the Secondary Chamber is bulging with Priming Solution, close the Secondary Chamber Line Clamp and the Priming Line Clamp. With a massaging action, dislodge any air bubbles that may be trapped in the corners of the Secondary Separation Chamber toward the Primary Line Coupler. Tapping the chamber with fingers may help in mobilizing the air bubbles.

Open the Priming Line Clamp, the Secondary Chamber Line Clamp and the Primary Line Clamp. Allow approximately 200 ml of Priming Solution to flow into the Primary Separation Container while ensuring that all air bubbles are removed from the Secondary Separation Chamber, Primary Line and Secondary Chamber Line. Close all clamps. At this time, the MaxSep Disposable Set is fully primed with the exception of the Cell Suspension Line and the 8-Couplers Manifold that will connect to the Cell Suspension Containers. Also, the Primary Separation Chamber contains air that will need to be expressed into one of the Cell Suspension Containers once they are connected to the 8-Couplers Manifold. Inspect the entire MaxSep Disposable Set for leaks, specially around SCD312 welds. If any leaks are found, consider the set contaminated and discard. Go back and set up a new disposable set.

On average, there will be eight 3-liter Lifecell Flasks inside the CO_2 Incubator containing the Cell Suspension to be processed. Remove two of them at a time and place them inside the Laminar Flow Hood. Verify the Cell Culture Identification Numbers against the ones recorded previously. Once the verification process is complete, remove the flask's spike port blue protective cover from one of the Cell

Suspension Containers and uncap one of the spike connectors from the 8-Couplers Manifold. Aseptically connect the two. Repeat this procedure with the other Cell Suspension Container inside the hood. Remove two more flasks from the incubator, repeat the Cell Culture Identification Number verification process and connect to the 8-Couplers Manifold. Repeat these steps until all the Cell Suspension Containers to be processed are connected to the Primary Separation Chamber via the 8-Couplers Manifold.

Position the IV pole close to the opening of the Laminar Flow Hood. Carefully move the Cell Suspension Containers from the hood to the IV pole making sure no tension is created on the tubing lines. Gather the rest of the MaxSep Disposable Set inside the Laminar Flow Hood in an organized manner to prevent the tubing from becoming entangled. Move the IV pole and the Disposable Set to the area where the MaxSep Separation System is located. The IV pole will be "top-heavy" so use extra caution when moving it to prevent the pole from tipping. Lay out the Disposable Set on the counter top where the MaxSep Separation System is located. Hang the Priming Solution Container vertically from the mini IV pole that is built into the MaxSep System. Position the Primary Separation Container so that the air inside the container is on the side where the spike ports are located. Roll up the container or place it in a Plasma Extractor.

While maintaining pressure on the Primary Container, open the Cell Suspension Line Clamp and one of the individual Lifecell Flask Clamps. Express the air out of the Primary Container and into the flask. Continue to force fluid out of the Primary Container until all air has been cleared and the line is filled with Priming Solution. Close the Lifecell Flask Clamp. Repeat these steps until all Lifecell Flask Lines are free of air and filled with Priming Solution. Ensure all clamps on the Disposable Set are closed. Place the Cell Recovery Containers approximately 6 inches below the level of the surface where the MaxSep Separator is located.

C. **Installation of Disposable Set on the Max Sep Separation System**

Retract the Locking Pins and open the Primary Magnet Door. Rotate the Port Restraining Lock 90° so that the Port Restraining Arm can be lifted to the open position. Place the Primary Separation Container squarely over the Primary Magnet that is in the horizontal (0°) position. Do not close the Primary Magnet Door at this time. Open the Secondary Magnet Door and place the Secondary Separation Chamber on the Secondary Magnet. Orient the Secondary Chamber Line so that it faces the front of the Magnetic Cell Separator and the Primary Line faces toward the back of the Magnetic Cell Separator.

Place the Secondary Chamber Line Bushing into the recessed area provided on the Secondary Magnet toward the front of the Separator. Next, place the Primary Container Line Bushing and tubing into the recessed area provided toward the back of the Separator. The Secondary Chamber should fit in the recessed area provided. Lower the Secondary Magnet Door over the Secondary Chamber. The door will not completely close at this time since the Secondary Chamber contains excess Priming Solution. Close and lock the Port Restraining Arm over the Primary Container ports to secure them. Rotate the Port Restraining Lock 90° to secure the Restraining Arm. The notched region of the Locking Block is for use with the 1000 ml container.

Back flush excess Priming Solution from the Secondary Separation Chamber into the Primary Container by opening the Primary Line Clamp. The magnetic force on the door over the Secondary Magnet will force the door to close and to squeeze fluid out of the Secondary Separation Chamber. When back flush is complete, close the Primary Line Clamp. Close the Primary Magnet Door by lifting from the hinged side and lowering the free end onto the Primary Container. While supporting the door, slide the two Locking Pins over the door to restrain it. Should this not be possible, the Primary Container is overfilled and fluid must be removed until door closure is possible.

Excess fluid from the Primary Separation Container can be removed by opening the Cell Suspension Line Clamp and one of the Lifecell Flask Clamps. The flask must be at a lower level than the Primary Container for fluid to flow in the direction of the flask. Close both clamps once door closure is achieved. Position the Primary Magnet at a 45° angle and gently tap the Primary Magnet Door to dislodge any air bubbles that may have formed during manipulation of the container. Any bubbles present must be at the end of the Primary Container that has no ports and as far away from the Primary Line Coupler as possible. Place the Primary Magnet at a 15° angle. Ensure all clamps are closed.

D. Separation Procedure

This is a modified magnetic bead removal procedure using the Baxter Fenwal MaxSep Magnetic Cell Separation System. The maximum flow rate using the peristaltic pump built into the MaxSep is 30 ml/min. In order to achieve higher fluid flow rates, the MaxSep's pump is bypassed and gravitational force is used to feed the fluid through the primary and secondary separation chambers and into the recovery containers. A semi-closed system has been developed to reduce the risk of exposure to biohazardous fluids. All other manufacturer's guidelines have been followed to the fullest extent possible.

Ensure that all the clamps on the MaxSep Disposable Set are closed. Adjust the height of the IV pole that holds the Cell Suspension Containers so that the top of fluid in the flasks is at 35 inches from the surface where the MaxSep Separator is located. This height will allow flow rates of approximately 180 ml/min. Open each of the following clamps in the specified order: 1) one of the Lifecell Flasks Clamps; 2) Cell Suspension Line Clamp; 3) Primary Line Clamp; 4) Secondary Chamber Line Clamp; and 5) Reservoir Bag Line Clamp. The timing of the opening of the clamps is very important. Each must be sequentially opened to prevent pressure buildup in the system. Excessive pressure increases the possibility of beads being washed downstream into the Recovery Containers.

Once separation has started, allow approximately 400 ml of the processed Cell Suspension to accumulate in the Reservoir Bag. Then, open the Cell Recovery Line Clamp and the Cell Recovery Containers Clamps. Filling of the Cell Recovery Containers may be done individually or simultaneously. Care must be exercised to prevent build up of back pressure within the tubing set when filling the Recovery Containers individually. This decreases Cell Suspension flow rates. If Recovery Containers are being filled simultaneously, attempt to maintain equal distribution of fluid in all containers. During the separation process, monitor the status of the Primary and Secondary Separation Chambers for signs of inadequate magnetic separation. If too many microbeads are accumulating at the Secondary Separation Chamber, perform a back flush of the Secondary Chamber using the Priming Solution. Occasionally a back flush of the Primary Separation Container may also be necessary.

To perform a Secondary Separation Chamber back flush, allow the Cell Suspension Container currently being processed to empty, and close the clamp to the container. Close the Cell Suspension Line Clamp. Do not close the Primary Line Clamp. Allow the Primary Separation Chamber to partially empty. Close the Primary Line Clamp, the Secondary Chamber Line Clamp and the Reservoir Bag Line Clamp. Open the Secondary Magnet Door and remove the Secondary Separation Chamber. Open the Priming Solution Line Clamp and the Secondary Chamber lamp. Allow the Secondary Chamber to fill with Priming Solution and close the Secondary Chamber Line Clamp. With a massaging action resuspend the microbeads inside the Secondary Chamber. Elevate the Secondary Chamber so that it is higher than the Primary Separation Chamber. Open the Primary Chamber Line Clamp and allow the fluid in the Secondary Chamber to flow into the Primary Chamber. Close the Primary Chamber Line Clamp.

Open the Secondary Chamber Line Clamp, allow the Secondary Chamber to fill with Priming Solution and close the Secondary Chamber Line Clamp. With a massaging action resuspend the microbeads inside the Secondary Chamber. Elevate

the Secondary Chamber so that it is higher than the Primary Separation Chamber. Open the Primary Chamber Line Clamp and allow the fluid in the Secondary Chamber to flow into the Primary Chamber. Close the Primary Chamber Line Clamp. Close the Priming Solution Container Clamp. Reposition the Secondary Chamber on the Secondary Magnet and close the Secondary Magnet door. If the door does not close completely, open the Primary Line Clamp to allow excess fluid in the Secondary Chamber to exit toward the Primary Separation Chamber. Resume the separation process.

Regularly agitate the Lifecell Flasks to resuspend the Cell Suspension and maintain appropriate cell and bead distribution. This should be done gently as not to create excessive foaming in the flasks. When the Lifecell Flask being processed is close to empty, it may be necessary to manipulate the flask to optimize cell recovery. To do this, tilt the Lifecell Flask so that the cell suspension is directed toward the port that is connected to the Cell Suspension Line. Close the empty Lifecell Flask Clamp before fluid level reaches the first "Y" juncture of the manifold to prevent air from entering the Primary Separation Container. Repeat these steps until all flasks are empty. Close the Cell Suspension Line Clamp when all flasks have been emptied. Closely watch the emptying of the Primary Separation Container. When all but a few milliliters of fluid are left in the Primary Container or bubbles begin to leave the Primary Container, close the Primary Line Clamp, the Secondary Chamber Line Clamp and the Reservoir Bag Line Clamp. Empty the contents of the Reservoir Bag into the Cell Recovery Containers. Attempt to clear the Cell Recovery Line of Cell Suspension by applying pressure to the Reservoir Bag to maximize cell recovery. Close the Cell Recovery Line Clamp and the Cell Recovery Container Clamps. Heat seal the Cell Recovery Line twice and detach from the Disposable Set. To detach the Cell Recovery Containers from the 8-lead manifold, heat seal each individual tubing lead twice close to the container (approximately 1"). The Cell Suspension is now ready for further processing as indicated. Dispose of the MaxSep Disposable Set per Infectious Waste Control Plan.

Any fluid leakage or disposable set disconnection constitutes a break in sterility and the set must be discarded and replaced. If this happens during the priming procedure, simply discard the faulty disposable set. Should the break in sterility occur during the actual magnetic cell separation process, all the clamps on the disposable set must be closed and the spill of fluid contained per standard guidelines.

EXAMPLE 11

Harvesting and Washing of CD 3 + 28 Product

The Fenwal Harvester® System is a system for performing rapid, continuous, centrifugal separation of large volume suspensions. The system consists of the Harvester and various disposable sets. The Harvester is manually operated by means of a control panel. Suspensions are processed within sterile disposable sets. The system is designed to collect either particulate concentrate, supernatant or both. The particulate phase of the suspension is centrifugally separated and collected within a belt-type centrifuge chamber while the liquid phase or supernatant is removed via a line for aseptic collection or disposal. During operation, various monitors continuously check for abnormal conditions. A combination of visual and audible alarms keep the operator informed of the operating status and any abnormal conditions.

Cell culture volumes average approximately twelve liters per individual culture once the cell expansion goal is reached. It is advantageous to efficiently reduce the cell containing media to a manageable volume both for handling and for transfusion purposes. The final component that is used for transfusion into the donor-recipient should be of a volume that will not cause fluid overload. At the same time, it is advantageous to achieve this volume reduction without incurring a significant loss of cells. The Baxter Fenwal Harvester efficiently accomplishes these goals.

Infusion of culture media into the recipient is undesirable as it is not an approved medium for intravenous use. Therefore, a wash step is instituted into this

procedure using one liter of 0.9% sodium chloride followed by a wash with one liter of Plasmalyte-A (an electrolyte solution). Plasmalyte-A is also the final cell suspension solution. Cells collected from HIV seropositive donors are contained in the Cell Suspension Containers that are to be processed. The Fenwal Harvester System and its sterile Disposable Sets provide a semi-closed system that protects the sterility of the cell cultures being harvested and reduces the risk of exposure to biohazardous fluids. Cell culture harvest is performed the day of scheduled cell reinfusion and once the cultures have undergone the magnetic cell separation process for removal of the microbeads used for cell stimulation.

A. Harvester Disposable Sets Installation

First, remove the Reservoir Set from the pouch. Close the two clamps on the Reservoir Set and place a Hemostat on each Inlet Line. Hang the Reservoir Bag from the hook located on the metal support bar, open the Supply Pump cover and lift the Rotor Locking Handle. Install the Pump Segment by placing the Segment Collar on the outside of the Pump Inlet (lower groove) following the arrows on the pump. Place the Segment between the Locator Rods of the pump and turn the Locking Handle counterclockwise to thread the segment through the pump. Complete the installation by placing the segment into the Pump Outlet groove and turning the handle until it locks. Close the Supply Pump Cover. Install the lower Pump Segment into the right side of the Pressure Monitor by stretching the tubing and inserting it into the groove. Remove the Harvester Set from the pouch. Fold the belt in half and roll up to evacuate any air. Close all clamps on the set.

Next, open the doors to the Centrifuge Compartment of the Harvester to gain access to the Belt Support. Remove the cover by turning the Locking Handles so that the red knobs are facing toward the center of the Belt Support. Remove the white Restraining Collar from the center of the Belt Support. Uncoil the three lines from the pack and insert them into the center of the Belt Support. Pull the lines out through the Guide Hole. Place the Belt Chamber on top of the Belt Support with the Belt Inlet (two tubing end) on the right and the Belt Outlet (three tubing end) on the left. Place

the Restraining Collar around the Lower Hex Strain Relief. Insert this assembly into the rotor and rotate until a distinct locking click is heard/felt. Pull the lines out of the Guiding Hole and up over the top door. Position from the left to right across the machine.

Then, place the straightened Multiple Lumen Tubing to the right of the Restraining Arm on the Rotor Shield. Rotate the Rotor Shield counter-clockwise until the Restraining Arm and Multiple Lumen Tubing are aligned on the right side of the Centrifuge Compartment. Align the Upper Hex Strain Relief on the Support Bar located above the Centrifuge. Close the Restraining Collar Latch. Insert the Multiple Lumen Tubing inside the Restraining Arm along the entire length of the arm. Make sure the tubing is straight (follow blue line on the side of the tubing). Slide the Multiple Lumen Tubing Retainer onto the open side of the Restraining Arm with the Baxter logo up.

Pull the Multiple Lumen Tubing to the left side of the Harvester, shape the tubing into a "U" and hold in the groove located toward the back of the top left side. Close the top door of the Harvester far enough to hold the tubing in place. Pull the excess length outside the Centrifuge Compartment. Separate the tubes as follows: the Inlet Line (it has two small tubes with a Pump Segment and a Coupler) lays across the top of the Harvester; the Harvest Line (one small tube) lays across the top/back of the machine and the Waste Line is off to the left side.

Open the Processing Pump cover and lift the Rotor Locking Handle. Install the Pump Segment of the Inlet Line by placing the Segment Collar on the outside of the Pump Inlet (upper groove) following the arrows on the pump. Place the segment between the Locator Rods of the pump and turn the Locking Handle counterclockwise to thread the segment through the pump. Complete the installation by placing the segment into the Pump Outlet groove and turning the Locking Handle until it locks. Close the Processing Pump cover. Install the Lower Pump Segment into the left side of the Pressure Monitor by stretching the tubing and inserting it into the groove.

Place a Hemostat on the large tubing of the Waste Line close to the connection site of the three small tubes.

Then, remove the Reservoir Bag Port protector and the Processing Pump Inlet Line Spike protector. Aseptically connect the two. Open the clamp that is between the Processing Pump and the Reservoir Bag. Open the Processing Pump cover, lift the Rotor Locking Handle and slowly turn clockwise to evacuate air from the belt into the Reservoir Bag. Turn the handle until it locks and close the pump cover. Position the Belt Inlet (two tubing end) at the "inlet" marking on the Belt Support. Lift the Plunger and insert the Belt into the slot around the perimeter of the Belt Support. Starting at the Belt Inlet, insert the belt at a shallow depth and continue in a counterclockwise motion around the belt slot. After the complete belt has been started into the slot, it can be easily pushed down until it is flush with the top surface of the Belt Support outer ring. Temporarily align the small tubing lines over the top of the Belt Support following their natural positioning. The three Outlet Tubing Lines are on the left and the two Inlet Tubing Lines are on the right. Ensure that the tubing lines are not kinked or crossed as they go down into the Belt Slot. Also, confirm that the tubing lines are not twisted or kinked where they come out of the Restraining Collar. Tape the five tubing lines to the Rotor so as to avoid any twisting or kinking during the operation of the Harvester. The tubing lines should lay flat against the Belt Support once they are taped down.

Replace the Belt Support Cover over the Alignment Pins and Plunger and lower into place. Check for an orderly, almost parallel tube alignment of the five small tubing lines as they are secured under the cover. The tubing lines may be manipulated for proper alignment by reaching through the center hole of the Belt Support Cover. Ensure that the tubing lines are not crossed or pulled too tightly as to cause kinking as they pass over the Belt Support edge going down onto the Belt Slot. Secure the cover by turning the Locking Handles to the locked position (red knobs facing outward). If resistance is felt, check to see if the tubing lines and the belt are

properly positioned. Attach the preassembled Waste/Harvest Containers to the Harvest Line by removing the tip protectors and spiking.

Preassemble the Waste/Harvest Containers as follows. Gather the Terumo SCD312 Sterile Connecting Device/Wafers (as needed), 1000 ml Lifecell Flask (1), 600 ml Transfer Pack (1), "Y" juncture from a 600 ml Transfer Pack with Eight Couplers (1), Plasma Transfer Set (1) and Sebra Dielectric Heat Sealer (1) inside a Laminar Flow Hood. Using the SCD312, attach the 1000 ml Lifecell Flask to one of the legs above the "Y" juncture. Close the clamp. Using the SCD312, attach the 600 ml Transfer Pack to the other leg above the "Y" juncture. Close the clamp. To the single leg below the "Y" juncture, connect a section of the Plasma Transfer Set with a spike connector at the opposite end using the SCD312 and close the clamp.

Remove the Waste Line end protector and one of the spike protectors of the Plasma Transfer Set and connect. Connect a 10-Prong Manifold to the other spike connector of the Plasma Transfer Set. Heat seal the tubing lines attached to the 2000 ml Transfer Packs twice and discard the tubing. Connect a 2000 ml Transfer Pack to each of the spike connectors on the 10-Prong Manifold. These will be the Waste Containers. Close all the clamps on the Manifold. Remove the tip protector from the other 10-Prong Manifold and from one of the Reservoir Bag Inlet Line spike connector and attach to each other. Close all the clamps on the 10-Prong Manifold. Keep the Hemostat in place. Remove the tip protector from the 5-Prong Manifold and from the other Reservoir Bag Inlet Line spike connector and attach to each other. Close all the clamps on the 5-Prong Manifold. Keep the Hemostat in place.

Before proceeding, the following conditions should exist: a) the Multiple Lumen Tubing should be straight and not twisted; b) the belt, associated tubing and cover should be in proper position and Cover Locking Handles should be in the locked position (red knobs out); c) Hex Strain Reliefs should be properly positioned in their respective Restraining Collars; d) the white Restraining Collar should be locked into place; e) the Multiple Lumen Tubing should be positioned completely inside the

length of the Restraining Arm; f) the Multiple Lumen Tubing Retainer should be properly positioned; g) the front and top doors to the Centrifuge Compartment should be completely closed; h) Hemostats should be placed as specified - the Waste Line on the large tubing area close to the connection site of the three small tubes, and Inlet Lines of the Reservoir Bag; and i) All the clamps on the disposable sets should be closed except for the clamp between the Reservoir Bag and the Processing pump which remains open.

B. Harvester Disposable Sets Priming Procedure

First, ensure that all the clamps are closed except for the clamp between the Reservoir Bag and the Processing Pump. Connect three 1000 ml 0.9% NaCl bags to the 5-Prong Manifold using the spike connectors. Turn the Harvester on by pressing the green power button on the Control Panel. The button should illuminate. Press the Centrifuge "fast" button. It should be programmed to 1600 rpm and the display should indicate 1600 rpm. If the Centrifuge is not spinning at 1600 rpm, adjust the fast rotor knob on the back of the machine until the display reads 1600 rpm. Refer to the Operator's Manual for further instructions on the adjustment of the Back Panel Controls. Open the Waste Line Clamp and the clamp to one of the 2000 ml Waste Containers. Do not remove the hemostat from the waste line. Open the NaCl bags' clamps on the 5-Prong Manifold, remove the Hemostat and open the clamp to the Reservoir Inlet Line connected to the 5-Prong Manifold. A Code 6 alarm should now be displayed; this indicates a low Reservoir and is normal at this time. Press the mute button to silence the audible alarm.

Next, pump approximately 500 ml of NaCl into the Reservoir Bag by pressing the F-JOG button of the Supply Pump. The LED display should indicate the rate at which the fluid is being pumped into the bag. The Supply Pump flow rate should be set at 700 ml/min. If the flow rate is other than 700 ml/min, adjust the pump speed. To adjust the Supply Pump flow rate, press the "fast" button of the Supply Pump, on the Harvester's Back Panel Controls, locate the "supply fast" knob, and adjust as necessary to attain a flow rate of 700 ml/min.

Then, press the "fast" button for the Processing Pump. The LED display should indicate a flow rate of approximately 600 ml/min. To adjust the Processing Pump flow rate, press the "fast" button of the Processing Pump (a Code 6 (low reservoir) condition should not exist, or the Processing Pump will not operate), then locate the "processing fast" knob on the Back Panel Controls, and adjust as necessary to attain a flow rate of 600 ml/min. The supply pump should be set at a faster flow rate than the processing pump. NaCl from the Reservoir Bag should be flowing into the centrifuge *via* the Multiple Lumen Tubing. Squeeze the Processing Line with fingers between the Processing Pump and the Reservoir Bag to free any air bubbles that may have formed in that area.

Wait for a Code 5 alarm which indicates that the fluid being pumped into the centrifuge has filled the Belt Chamber and is ready to exit *via* the Waste Line. This alarm condition causes the Processing Pump and the Centrifuge to stop. Remove the Hemostat from the Waste Line. The machine should return to normal operation (Processing Pump and Centrifuge restart) once the Hemostat is removed since the build up of pressure has been resolved. Alternately pinch two of the three small tubing lines exiting the Centrifuge Compartment with the Hemostat to clear the lines of any trapped air inside the Belt Chamber. Check the flow of fluid on the Waste Line. It should be a smooth flow at the approximate programmed processing rate. Check to see if any air bubbles have formed in the Inlet Lines by pressing and holding the strobe button. If there are any, try to free them by pinching one of the Inlet Multiple Lumen Tubes.

If there are no problems at this point, press the off buttons of the Processing Pump and Supply Pump. Keep the Centrifuge running and clamp the Waste Line with a Hemostat. Close the clamp to the 2000 ml Waste Container that collected the priming solution as it should be almost full. Close the clamps to the NaCl bags and the clamp on the Reservoir Inlet Line connected to the 5-Prong Manifold. Hemostat the Inlet Line. The Harvester is now ready to start processing the Cell Culture.

C. **Cell Culture Harvest With Wash**

During alarm conditions or in any other situation where the centrifuge slows down, stops or must be stopped, close the clamp on the Waste Line and apply a Hemostat to the line. This is to prevent accidental siphoning of cells from the Belt Chamber into the Waste Containers.

First, verify the Identification Numbers on each of the Cell Culture Containers to ensure that the correct cell cultures are being processed. In addition to verifying the Cell Culture Identification Numbers, ensure that the Cell Culture Label specifies that the culture has undergone the Magnetic Separation Process. Using the 10-Prong Manifold, spike one of the Cell Culture Containers. Open the clamp to that Culture Container, the clamp on the Reservoir Inlet Line and remove the Hemostat. Press the "fast" button for the Supply Pump. If the LED display does not remain lit and the pump stops once the button is released, it indicates that the Reservoir Bag has low volume. Code 6 should also be displayed at this time. To resolve this condition, keep the "fast" button depressed until the LED display shows the pump speed and then release.

Next, remove the Hemostat from the Waste Line and open the clamp to one of the empty Waste Containers. Continuously monitor the status of the Waste Containers for overfilling. Close and open Waste Containers as needed during the procedure. Press the "fast" button for the Processing Pump. The Cell culture in the Reservoir Bag is now being pumped into the Belt Chamber and harvest has started. Ensure that the Supply Pump flow rate is 700 ml/min and the Processing Pump is 600 ml/min. When the Cell Culture Container being processed is close to empty, connect another container to the 10-Prong Manifold. When the container being processed empties, close its clamp and open the clamp to the next Culture Container.

Continue to connect Cell Culture Containers to the manifold as the ones being processed empty. Always clamp the appropriate Cell Culture Container line clamp

when it empties and open the clamp to the full container that is to be processed next. This ensures that if there is a malfunction or problem during the harvest procedure which compromises the sterility of the cell cultures, not all the containers will be affected. Periodically re-suspend the cells by manually shaking the Culture Containers. Monitor the progress of the harvest process; occasionally press the strobe button and check the Centrifuge Compartment for any signs of improper operation or malfunction.

After all the Cell Culture Containers have been processed, connect a 1000 ml bag of 0.9% NaCl and a 1000 ml bag of Plasmalyte-A to the 5-Prong Manifold. Do not open the clamps. These solutions will be used for the Wash step. Once the Cell Culture Containers are emptied and the last of the cells are in the Reservoir Bag, press the off button for the Supply Pump. Close the clamp on the Reservoir Bag Inlet Line that connects to the 10-Prong Manifold and place a Hemostat on the line. At this point, a CODE 6 alarm may sound. At this point, the Reservoir Bag is low and the Processing Pump automatically stops. Press the mute button to silence the alarm. Press the F-JOG button for the Processing Pump until the Reservoir Bag is almost empty and then release. Do not allow air to enter the processing line (left side leading into the centrifuge). There should be enough 0.9% NaCl left in the three bags connected to the 5-Prong Manifold which initially were used to prime the Harvester. If less than 1000 ml of NaCl is left (combine the volumes in each bag), connect another bag. Remove the Hemostat and open the clamp on the Reservoir Bag Inlet Line that connects to the 5-Prong Manifold.

Press the F-JOG button for the Supply Pump and allow approximately 300 ml of NaCl to enter the Reservoir Bag and then release. Massage the Reservoir Bag to rinse. Press the F-JOG button of the Processing Pump until the Reservoir Bag is almost empty. Once again, do not allow air to enter the Processing Line. Repeat these steps twice. Once completed, close the clamps to the 0.9% NaCl bags used for these steps. Open the clamp to the full bag of 0.9% NaCl and press the "fast" button for the Supply Pump until the machine automatically takes over filling the Reservoir

Bag. Press the off button for the Supply Pump once the bag empties and close its clamp.

Then, press the "slow" button for the Supply Pump so it is operating at a flow rate of 300 ml/min. If the LED display indicates a different flow rate, adjust it using the Back Panel Controls (use the processing slow knob to regulate the pump speed). Allow the NaCl to run through the machine. When the low Reservoir alarm sounds (Code 6), press the Processing Pump off button and the mute button. Open the clamp to the 1000 ml bag of Plasmalyte-A. Press the "fast" button for the Supply Pump until the machine automatically takes over filling the Reservoir Bag. Press the off button for the Supply Pump once the bag empties. Close the clamp to the Plasmalyte-A bag and the clamp on the Reservoir Bag Inlet Line. Place a Hemostat on the line. Press the "slow" button for the Supply Pump so it is operating at a flow rate of 300 ml/min. Allow the solution to run through the machine. When the low Reservoir alarm sounds (Code 6), press the Processing Pump off button and the mute button. Place a Hemostat on the Waste Line close to the point where the three small tubing lines connect. Close the Waste Line Clamp. Press the off button for the Centrifuge.

Open the Supply Pump compartment cover and remove the tubing by lifting the Locking Handle and turning the rotor. Install the Harvest Line on the Supply Pump (follow the same steps used for the installation of the pump segment of the Inlet Line - Refer to Section A above). Open both the small and large clamps on the Harvest Line. Open the roller clamp to the Waste Container (600 ml Transfer Pack) connected to the Harvest Line.

Open the top door to the Centrifuge Compartment. Remove the Belt Support Cover (rotate the Locking Handles so that the red knobs face toward the center of the Belt Support). Ensure that all five small tubing lines that enter and exit the Belt Chamber are visible. Press the Supply Pump F-JOG button while watching the three outlet lines closely to ensure that only Plasmalyte-A solution exits the belt. If cells are seen in the outlet lines of the belt, release the F-JOG button. This step is only

used to siphon out excess Plasmalyte-A solution; no cells should exit the Belt Chamber at this time. Close the roller clamp to the Waste Container connected to the Harvest Line. Open the roller clamp to the Harvest Container (1000 ml Lifecell Flask).

Lift the Belt Support Plunger and carefully remove the Belt Chamber from the perimeter of the Belt Slot. Ensure that no lines are pinched in the removal process. Do not remove the white Restraining Collar at this time. Use the back of one hand to rub up and down the Belt Chamber until all the cells are resuspended. Any other form of manipulation of the belt can be used to achieve the best possible resuspension of cells as long as it does not compromise the patency of the Belt Chamber. With one hand hold the Belt Chamber so that the three Outlet Tubing Lines are at the bottom. Press the Supply Pump F-JOG button so that the cells flow from the Belt to the Harvest Container. Release the F-JOG button once a vacuum is created. Add some Plasmalyte-A solution from the Reservoir Bag to the Belt Chamber by pressing the Processing Pump F-JOG button and then releasing it. Then repeat the cell resuspension procedure.

Next, empty the contents of the Belt into the Harvest Container as described above. Repeat both the addition of Plasmalyte-A solution from the Reservoir Bag to the Belt Chamber and then pressing the Supply Pump F-JOG button so that the cells flow from the Belt Chamber to the Harvest Container until all of the cells have been cleared from the Belt Chamber (two or three Belt rinses should be sufficient). Remove the Reservoir Bag from the support and invert it. Press the Processing Pump F-JOG button until the Belt Chamber starts to fill with air. Hold the Belt up with the three Outlet Tubing Lines at the bottom. Press the Supply Pump F-JOG button and allow the air inside the Belt to clear the lines all the way to the Harvest Container. Close the roller clamp to the Harvest Container. Heat seal the Harvest Container Line twice as close to the "Y" juncture as possible and detach. Affix a Cell Culture Identification Label to the Harvest Container.

Inside the Laminar Flow Hood, connect the other 1000 ml Lifecell Flask to the Harvest Container. This flask will be the final component container. Ensure that the roller clamp remains part of the setup. Place the Harvest Container flat (0° position) on the Primary Magnet of the Baxter Fenwal MaxSep Magnetic Cell Separation System. Close the Primary Magnet door and allow the Harvest Container to rest on the magnet for approximately one minute. This is to trap any microbeads that may have escaped the initial magnetic separation process. Place the Primary Magnet on a vertical position (90° position). Open the roller clamp between the Harvest Container and the final component container (1000 ml Lifecell Flask). Allow the Harvest Container to completely empty and close the roller clamp. Heat seal the tubing between the two containers three times and detach. Ensure that at least two heat seals remain on the tubing line connected to the final component container.

Affix an Identification Label to the Lifecell Flask that contains the cells. This label should include the date, donor information, recipient information, the phrase "for autologous use only", the processes the cells have undergone to this point, the suspension solution used, and a biohazard symbol. Place the final component container inside a leak-proof transporting container. Remove Disposables from the Harvester and Disinfect Harvester with a 50% Sodium Hypochlorite/water solution.

Any fluid leakage or disposable set disconnection constitutes a break in sterility and the set must be discarded and replaced. If this happens during the priming procedure, simply discard the faulty disposable sets and install new sets. Should the break in sterility occur during the actual harvest process and not inside the Centrifuge Compartment of the Harvester, stop the Harvester pumps, clamp and Hemostat the Waste Line, stop the centrifuge, close all the clamps on the disposable sets and contain the fluid spill. Should a break in sterility occur inside the Centrifuge Compartment during the harvest process, the spill of fluid should trigger a Code 7 alarm. This alarm condition shuts down all the Harvester's operations until the condition is corrected and the mute button is depressed. All personnel in the

processing room should leave the premises immediately to prevent exposure to any aerosol created by the spill.

Cells collected and expanded from HIV seropositive individuals are contained in the Cell Culture Containers; therefore, Universal Blood and Body Fluid Precautions should be strictly practiced at all times. During all steps of the procedure in which the Cell Culture is handled, gloves and disposable lab coats should be worn. If there is a possibility that a splash or aerosol may occur, goggles and/or face shields should be worn.

EXAMPLE 12

Procedure for the Infusion of CD3+28 T Cells

This procedure may be used for the removal of magnetic beads from cultured CD3+28 T cells and concentration of cells on the Fenwal CS3000+, release criteria for the final cell product must be established before cell infusion. The following materials were used: Coulter Multisizer IIe; Trypan Blue 0.4% Solution, (Sigma Chemical, St. Louis, MO); Plasmalyte A with 1% Human Serum Albumin; Albumin 25% solution; and Gram Stain Kit, (Sigma).

Following cell culture and harvest with wash as detailed above, an aliquot of cells is removed from the final product bag. Cells are counted and electronic volume determination performed on the Coulter Multisizer IIe. Cell viability may be assessed by Trypan Blue staining. Viability should be 80% or higher. Culture sterility can be assessed by referring to the microbiology reports from the cultures performed on day -2. To further assure sterility, a gram stain may be done and subjected to examination by microscopy. The gram stain report should report no organisms seen, and the bacterial, mycoplasma and fungal cultures from all previous culture reports, including day -2 should report no growth. The endotoxin assay from day -2 should have a result of <1 EU/ml/.

Adequacy of magnetic bead removal may be assessed by microscopy according to the following protocol: 1) 1×10^6 cells are added to a 1.5 ml microfuge tube in 3 replicates. Thus, the residual beads contained in 3×10^6 cells are assessed; 2) The cells are lysed by addition of sufficient Triton X-100 to bring the final concentration to 1%; 3) The beads are pelleted by microfuge centrifugation, and the supernatant aspirated, being careful to leave a nearly dry pellet containing about 50µl residual volume in each tube; 4) Use a pipet to gently resuspend the 50 µl, being careful not to create bubbles. Add the entire 50 µl to a microscope slide. Add a coverslip and scan the entire field for beads. Record the total number of residual beads; and 5) If more than 100 beads are observed on the three slides, repeat magnetic bead depletion by passing the final product through the magnetic field on the MaxSep device. Repeat the magnetic bead quantitation protocol in steps 1 through 4. The bead removal release criterion for the final product should be less than 100 beads per 3×10^6 cells.

Contingent upon acceptable results from the above release criteria, the cells are infused. Depending on the number of cells harvested and the target infusion number for a particular protocol, excess cells may be removed from the final bag and cryopreserved. The cells are thoroughly suspended in Plasmalyte A containing 1% human serum albumin in a final volume of 100 to 200 ml. The cells are reinfused unfiltered to the patient over 20 to 30 min and the patient should be monitored for adverse reactions as described herein. A leukocyte-reduction filter should not be used during administration of the final product. The cells should not receive gamma irradiation or be passed through x-ray irradiation devices designed to detect metal objects. Record the final disposition of the final product and any occurrence of adverse reaction.

In rodents, infusions of 9.6×10^4 beads/kg were not toxic and the beads could not be detected in tissue sections. In rodents given infusions of 8.3×10^8 beads/kg no toxicity was observed and beads were observed phagocytosed in cells of the reticuloendothelial system. The residual beads in the final product may be quantified

by measuring the beads in 3×10^6 cells. If <100 beads/3×10^6 cells are observed, then infusions of 3×10^{10} cells in a 70 kg patient would represent $< 1 \times 10^6$ total beads or $<1.43 \times 10^4$ beads/kg. Proper coordination between the clinical facilities and the laboratory is advantageous throughout the harvest and infusion process. "Controlled haste" is advantageous once cells have been harvested and removed from the cytokine containing culture medium. The time from cell concentration to beginning cell infusion should not exceed 1 hour. The final product should not be infused if more than 4 hours elapses after harvest.

EXAMPLE 13

Obtaining Cell Count And Size

To obtain a cell count and size using the Coulter Multisizer II Counter, first run Isoton (blank) as outlined in calibration procedure above to achieve a background of <100. Next, gently invert sample 3-4 times to evenly distribute cells. Then, add 10 ml Isoton into empty sample vial. Next, add 0.05 ml sample into sample vial. Then, gently invert sample vial 3-4 times to evenly distribute cells. Next, making sure probe is not clogged, place sample vial under probe and run to get cell count and size. Then, open Multisizer II software on computer. Press print button on Multisizer II to transfer data to the computer. Analyze with software. Refer to Coulter Multisizer AccuComp Color Software manual. Finally, rinse orifice of Sampling Stand with Distilled water over a waste bucket after running sample.

EXAMPLE 14

Preparing the Coulter Multisizer II with Sampling Stand

To calibrate the Coulter Multisizer II with Sampling Stand, turn on the Coulter by setting the power switches of the Multisizer II and associated Sampling Stand to on. Check to make sure Isoton bottle is properly sealed and full. Empty waste flask if necessary. Allow Coulter to warm for approximately 10 minutes.

Then, prepare Coulter controls. Refer to Coulter Multisizer II reference manual in order to set up controls properly. Add 10 ml Isoton to a vial. Add 1 drop of the appropriate Coulter Size Standards into vial. Gently invert sample 3-4 times to evenly distribute cells. Making sure probe is not clogged, place sample vial under probe. Making sure the instrument control is in the syphon mode, run calibration vial by first pressing the reset button on the Multisizer. Then turn the upper stopcock a quarter turn in the clockwise direction. The light in the Sampling Stand should turn on and the manometer height should increase. Wait a few seconds for the green light screen to indicate the presence of cells, then turn the upper stopcock another quarter turn in the clockwise direction to begin. The count is complete when the light turns off and the Multisizer II beeps. Calibration is complete if all expected value ranges are met. Run Isoton through system before beginning sample analysis.

EXAMPLE 15

Determining the Amount of Endotoxin Present in the Cell Culture

The equipment and materials include the sample (pH 7-8), culture sample, 1:10 dilution of culture sample heated at 70°C for five minutes, Stop Reagent (25% v/v glacial acetic acid in water), Limulus Amebocyte Lysate Test Kit, Chromogenic Substrate and Chromogenic Limulus Amebocyte Lysate (LAL).

Reconstitute *E. coli* Endotoxin by adding 1.0 ml of Limulus Amebocyte Lysate (LAL) Reagent Water warmed to room temperature. The actual concentration is determined by the value stated on the enclosed certificate of analysis. Shake vigorously for at least 15 minutes with a vortex mixer. This stock solution is stable for one month at 2-8°C. Prior to use, the solution should be warmed to room temperature and vigorously mixed for 15 minutes. This is advantageous because endotoxin tends to attach to glass.

To prepare the four standard endotoxin solutions, prepare a solution containing 1.0 EU/ml endotoxin by diluting 0.1 ml of the endotoxin stock solution with (x-1)/10 ml of LAL Reagent Water in a 6 ml Falcon tube, where x equals the concentration of the endotoxin vial (found in the certificate of analysis). This solution should be vigorously vortexed for at least 1 minute before proceeding.

Transfer 0.5 ml of this 1.0 EU/ml solution into 0.5 ml of LAL Reagent Water in a 6 ml Falcon tube and label 0.5 EU/ml. This solution should be vigorously vortexed for at least one minute before use. Next, transfer 0.5 ml of the 1.0 EU/ml solution into 1.5 ml of LAL Reagent Water in a 6 ml Falcon tube and label 0.25 EU/ml. This solution should be vigorously vortexes for at least one minute before use. Finally, transfer 0.1 ml of the 1.0 EU/ml solution into 0.9 ml of LAL Reagent Water in a suitable container and label 0.1 EU/ml. This solution should be vigorously vortexes for at least 1 minute prior to use.

Reconstitute one vial of 7 mg lyophilized Chromogenic Substrate by adding 6.5 ml of LAL Reagent Water to yield a concentration of approximately 2 mm. Protect Substrate from long-term exposure to light. Reconstitute LAL immediately before use with 1.4 ml LAL Reagent Water. Swirl gently to avoid foaming.

The addition of all reagents in the Limulus assay should be consistent. All tubes or Microplate wells should be treated in exactly the same manner in order to determine the proper endotoxin concentration. Reagents should be pipetted in the same order from well to well, and at the same rate. Pre-equilibrate the Microplate at 37°C±1.0° in the Microplate Reader. While leaving the Microplate at 37°C±1.0°C, carefully dispense 50 µl of standard or sample into the appropriate Microplate well. Each series of determinations should include a blank plus the four endotoxin standards run in duplicate. The blank wells contain 50 µl of LAL Reagent Water instead of sample. All reagent additions and incubations times are identical.

At time T=0, add 50 µl of LAL to the first Microplate well. Begin timing as the LAL is added. Once the LAL has been dispensed into all Microplate wells containing samples or standards, mix in Microplate Reader by remote control with SpectraMax software. At T=10 minutes, add 100 µl of substrate solution (prewarmed to 37°C±1.0°C). Pipette the substrate solution in the same manner as previously. Once the substrate solution has been dispensed into all microplate wells containing samples or standards, mix in Microplate Reader by remote control with SpectraMax software. At T=16 minutes, add 100 µl of stop reagent. Pipette the substrate solution in the same manner as previously. Once the substrate solution has been dispensed into all microplate wells containing samples or standards, mix in Microplate Reader by remote control with SpectraMax software. Read, analyze, and graph the absorbance of each microplate well at 405 nm with SpectraMax software.

EXAMPLE 16

Sample Analysis for Endotoxin Control

This procedure describes a method of using the SpectraMax 340 and SOFTmax PRO software to determine the amount of endotoxin present in the cell culture. The equipment and materials include: SpectraMax 340 Microplate Reader; SOFTmax PRO software; and Blank, Standards and Samples prepared in microplate.

Open SOFTmax PRO in the MS-DOS system of the computer. (The computer may have to be restarted in order to enter MS-DOS). Under the FILE menu, open the file named "template.pda". This can serve as the template for all samples. Check to make sure the SpectraMax icon in the top left corner of the Status Bar does not have a red "X" on top of it. If an "X" appears on top of it, the computer is not in contact with the instrument, so make sure the SpectraMax is turned on. Check the current temperature of the chamber of the SpectraMax. The Current Chamber Temperature Display is located to the right of the SpectraMax icon in the Status Bar. The temperature may be adjusted and/or turned on or off by clicking the Incubator button located to the right of the READ icon.

Open the drawer of the SpectraMax 340 by clicking on the Drawer button, the right button in the SpectraMax Status Bar. Place the microplate containing the Blank, Standards and Samples in the drawer. Close the drawer by clicking the Drawer button again. The Automix button may be used whenever the SpectraMax 340 and SOFTmax PRO software are on. During the Endotoxin experiment, the Automix button may be used at T=0, T=10 and T=16. It is recommended that the Automix also be used immediately prior to reading the microplate. In the Introduction section, enter the Patient Number in the line designated. In the Plate section, click the Template button located in the Status Bar. The Blank, Standards, and first 10 Bags and their 1:10 dilutions have been assigned wells. Delete or add wells if necessary. Delete wells by highlighting all undesired wells. Under the Group Pop-Up Menu in the Template Editor Tool Bar, click Clear. The SpectraMax 340 will no longer read these wells. Repeat if necessary. Add wells by highlighting the well desired. Under the Group Pop-Up Menu in the Template Editor Tool bar, click New. In the Group Settings window that appears, add the Name of the sample and, if necessary, select Unknown(Dilutions) under the Column Format section for Bag dilutions of 1:10. Click OK when finished in the Group Settings window. Repeat if necessary.

Print a copy of the Template by clicking the Print button in the bottom left corner of the Template window. Exit the Template window by clicking OK in the bottom right corner. Delete all unnecessary active Bag Bars. First highlight the undesired active Bag Bar. Then, under the Edit menu, click "'Delete Bag#". Repeat if necessary. Delete or add Plots for the Bags. Scroll to the Graph Section Tool Bar. Delete Plots for unnecessary Bags by clicking on the Plots button in the Graph Section Tool Bar. Highlight the undesired Plot(s). Click the Delete button. When finished, click the OK button. Add Plots for new Bags by clicking on the Plots button in the Graph Section Tool bar. Click on the New button in the top right corner of the Plots window. Identify the new Bag number and X- and Y-axis labels by clicking on the Pop-Up menus located to the right of the labels Group, X and Y, respectively. Click OK after each new Bag to enter it into the computer. Repeat if necessary.

When the microplate is ready to be analyzed, click the Read button in the SpectraMax Status Bar to start the reading. If further analysis is necessary, refer to the SOFTmax user manual. Universal blood and fluid precautions should be strictly practiced at all times.

EXAMPLE 17

Detecting Mycoplasmas In Cell Culture

First, prepare fresh cell cultures by centrifuging 0.5 ml of sample at 12,000 × g for 10 minutes at 4°C. Discard 400 ml of the supernatant, leaving 100 ml of the medium and the cell pellet in the tube. Using a pipette, resuspend the cell pellet with the remaining 100 ml of medium. Pipette gently to avoid generating the bubbles. The 100 ml cell suspension is ready for PCR.

For the first stage of the nested PCR, prepare the following reagents for each PCR tube: 10X PCR buffer, 5 ml; 1st stage primer mixture, 1 ml; dNTP, 1 ml; $MgCl_2$, 1 ml; Taq DNA polymerase, 0.2 ml; Distilled water to 45 ml. For each set of unknown samples tested, a minimum of two positive DNA controls and one negative control should be included. For the sample, pipette 5 ml sample to each sample tube, making a final volume of 50 ml. For the positive controls, pipette 5 ml of each positive control DNA into separate positive control tubes. For the negative control, pipette 5 ml of sterile distilled water into this sample tube. Place all tubes into the thermal cycler and perform the amplification reaction using the following program: Denaturation: Step 1: 94°C for 30 sec; Denaturation: Step 2: 94°C for 30 sec; Annealing: Step 3: 55°C for 2 min; Extension: Step 4: 72°C for 2 min; Final Extention: Step 5: 72°C for 5 min; repeat steps 2-4 for 30 cycles.

For the second stage of PCR, prepare the following reagents for each reaction: 10X PCR buffer, 5 ml; 1st stage primer mixture, 1 ml; dNTP, 1 ml; $MgCl_2$, 1 ml; Taq DNA polymerase, 0.2 ml, Distilled water 49 ml. Add the sample by carefully

pipetting 1 ml from the first stage PCR reaction tube into the second stage reaction tube. Repeat the amplification reaction of this protocol as shown above.

Then perform gel analysis and interpretation of PCR results. Mix 10 ml of the second stage PCR products with an appropriate amount of loading buffer and add to the sample wells of a 1.2% agarose gel stained with ethidium bromide. Use a 100 bp DNA ladder as a size marker. Run the gel and then view the results with a UV light box. Mycoplasma species commonly encountered as cell culture contaminants (according to the ATCC Mycoplasma PCR detection kit) should generate a second stage PCR DNA amplicon that ranges in size from 236-365 bp. They should be compared to *A. laidlawii*, which generates two DNA amplicons of 426-219 bp, the positive control, *M. pirum*, which generates a DNA amplicon of 323 bp, and the negative control, which should not contain any amplicons.

EXAMPLE 18

Monocyte Depletion

This procedure describes a method of depleting the amount of monocytes present in the peripheral blood mononuclear cell (PBMC) product prior to cell culture. The equipment and materials include: PBMCs in X-VIVO 15® with 5% autologous human serum; Falcon Vented Flask, 175 cm^2, straight neck; 37°C Incubator; Sterile Gloves; and Sterile Individually Wrapped Pipettes

First, count cells using a Coulter Multisizer IIe as described herein above.

Then, phenotype cells to determine the proportion of CD3/4, CD3/8, CD19 and CD45/14 cells in the PBMC product. Add PBMCs at 1×10^8 cells to each 175 cm^2 Falcon Flask. The cells should be in a volume of 20-40 ml. Place each Falcon Flask in 37°C incubator with 5% CO_2. Lie each flask on its side to ensure optimal cell adhesion to the plastic of the flask. Incubate for 2 hours at 37°C with 5% CO_2. Gently remove supernatant containing desired cells from each flask. Monocytes may be seen macroscopically sticking to the inside surface of flask. Phenotype product to

determine the degree of monocyte depletion and the composition of the final PBMC product. Count cells using a Coulter Multisizer IIe to determine the recovery rate, as described herein. Universal blood and fluid precautions should be strictly practiced at all times.

All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A method of treating a mammal with an immunoresponsive cancer, comprising:

a) obtaining a population of peripheral blood mononuclear cells from said mammal;

b) activating said population of peripheral blood mononuclear cells outside of said mammal to obtain a population of T cells that have been activated outside of the mammal; and

c) administering said population of T cells to said mammal, thereby inducing an immune response and treating a mammal with an immunoresponsive cancer.

2. The method of claim 1, wherein said mammal has lymphoma, multiple myeloma, renal cancer, ovarian cancer, prostate cancer, low-grade lymphoma, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), sarcoma, lung cancer, an opportunistic malignancy or melanoma.

3. The method of claim 2, wherein said mammal has lymphoma.

4. The method of claim 3, wherein said mammal has non-Hodgkin's lymphoma.

The method of claim 1, wherein said activation of said population of peripheral blood mononuclear cells comprises contacting said population of peripheral blood mononuclear cells with at least a first antibody and at least a second antibody.

6. The method of claim 5, wherein said at least a first antibody and said at least a second antibody are linked to a magnetic bead.

7. The method of claim 5, wherein said at least a first antibody and said at least a second antibody are distinct antibodies.

8. The method of claim 5, wherein said at least a first antibody is an anti-CD3 antibody.

9. The method of claim 8, wherein said at least a first antibody is an OKT3 antibody.

10. The method of claim 5, wherein said at least a second antibody is an anti-CD28 antibody.

11. The method of claim 5, wherein said at least a first antibody is an anti-CD3 antibody and said at least a second antibody is an anti-CD28 antibody.

12. The method of claim 1, wherein said population of T cells comprises CD4+ T cells.

13. The method of claim 12, wherein said population of T cells comprises predominantly CD4+ T cells.

14. The method of claim 13, wherein said population of T cells are CD4+ T cells.

15. The method of claim 1, wherein said population of T cells comprises CD4+ T cells and CD8+ T cells.

16. The method of claim 1, wherein said population of T cells are not activated upon administration to said mammal.

17. The method of claim 1, wherein said immune response comprises the production of T cells in said mammal.

18. The method of claim 17, wherein said immune response comprises the production of CD8+ T cells in said mammal.

19. The method of claim 1, wherein said population of T cells administered to said mammal are predominantly CD4+ T cells, and said immune response comprises the production of CD8+ T cells in said mammal.

20. The method of claim 19, wherein said population of T cells are predominantly CD4+ T cells, and said immune response comprises the production of CD8+ T cells directed against said immunoresponsive cancer in said mammal.

21. The method of claim 1, wherein said mammal is a human.

22. The method of claim 1, further comprising immunosuppression of said mammal prior to administration of said population of said T cells to said mammal.

23. The method of claim 22, wherein said immunosuppression comprises administration of chemotherapy, azathioprine, cyclosporine A, FK506, purine analogs, alkylating agents, or anti-T cell monoclonal antibodies to said mammal.

24. The method of claim 1, further comprising administering a stem cell transplant to said mammal.

25. The method of claim 24, wherein said stem cells comprise CD34+ cells.

26. A method of treating a mammal with non-Hodgkin's lymphoma, comprising:

a) obtaining a population of peripheral blood mononuclear cells from said mammal;

b) activating said population of peripheral blood mononuclear cells outside of said mammal to obtain a population of T cells that have been activated outside of the mammal; and

c) administering said population of T cells to said mammal, thereby inducing an immune response and treating a mammal with non-Hodgkin's lymphoma.

27. The method of claim 26, wherein said activation of said population of peripheral blood mononuclear cells comprises contacting said population of peripheral blood mononuclear cells with at least a first anti-CD3 antibody and at least a first anti-CD28 antibody.

28. The method of claim 27, wherein said at least a first anti-CD3 antibody and said at least a first anti-CD28 antibody are linked to a magnetic bead.

29. The method of claim 26, wherein said population of T cells comprises predominantly CD4+ T cells.

30. The method of claim 26, wherein said mammal is a human.

31. A method of treating a mammal with T cells that have a defective cytokine profile, comprising:

a) obtaining a population of peripheral blood mononuclear cells from said mammal;

b) activating said population of peripheral blood mononuclear cells outside of said mammal to obtain a population of T cells that have been activated outside of the mammal; and

c) administering said population of T cells to said mammal, thereby treating a mammal with T cells that have a defective cytokine profile.

32. The method of claim 31, wherein said activation of said population of peripheral blood mononuclear cells comprises costimulation with a population of anti-CD3 antibodies and a population of anti-CD28 antibodies.

33. The method of claim 32, wherein said population of anti-CD3 antibodies and said population of anti-CD28 antibodies are linked to magnetic beads.

34. A method of inducing lymphocytosis in a mammal, comprising:

a) obtaining a population of peripheral blood mononuclear cells from said mammal;

b) activating said population of peripheral blood mononuclear cells outside of said mammal to obtain a population of T cells that have been activated outside of the mammal; and

c) administering said population of T cells to said mammal, thereby inducing lymphocytosis in said mammal.

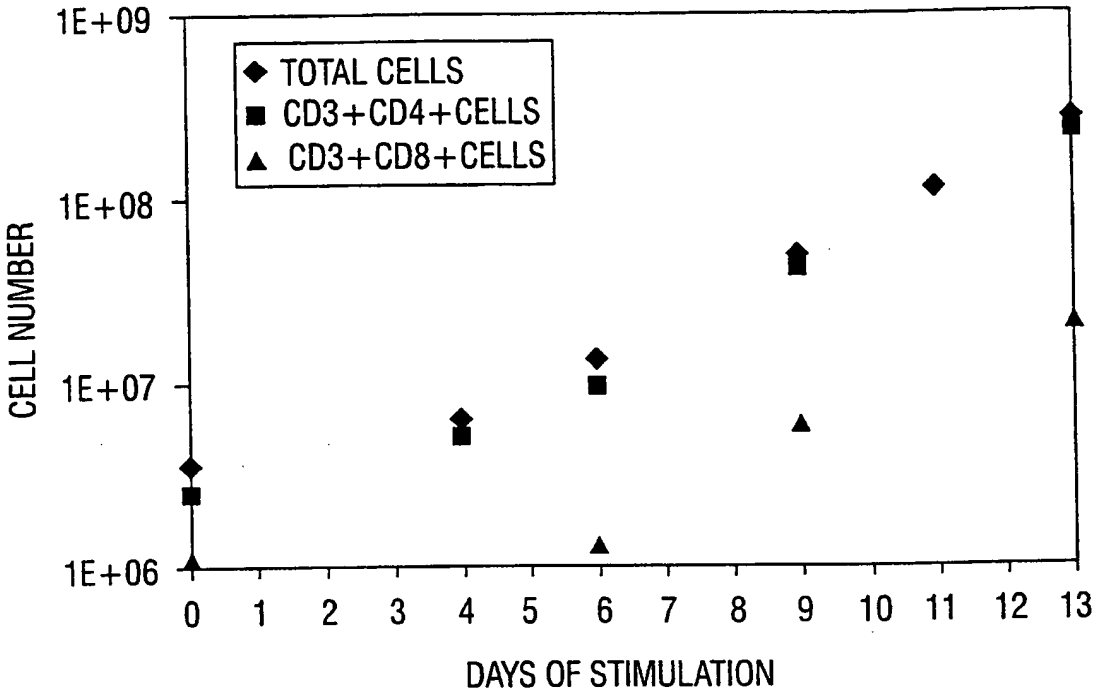

1E+09
1E+08
1E+07
1E+06
CELL NUMBER
TOTAL CELLS
CD3+CD4+CELLS
CD3+CD8+CELLS
0 1 2 3 4 5 6 7 8 9 10 11 12 13
DAYS OF STIMULATION

FIG. 1A

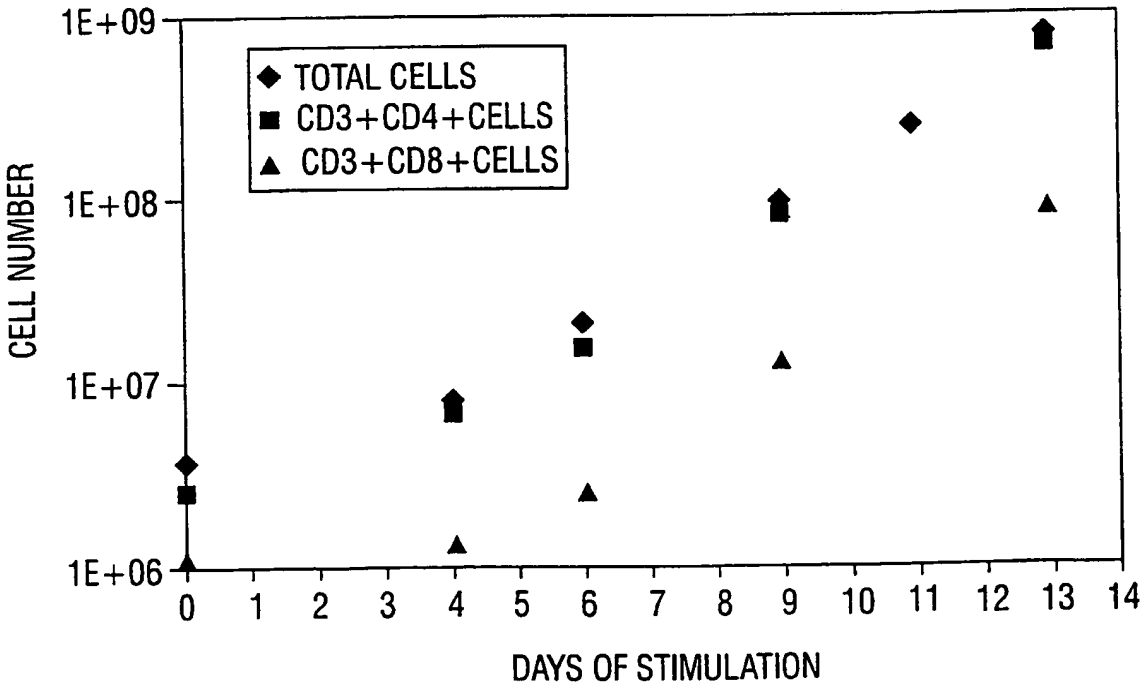
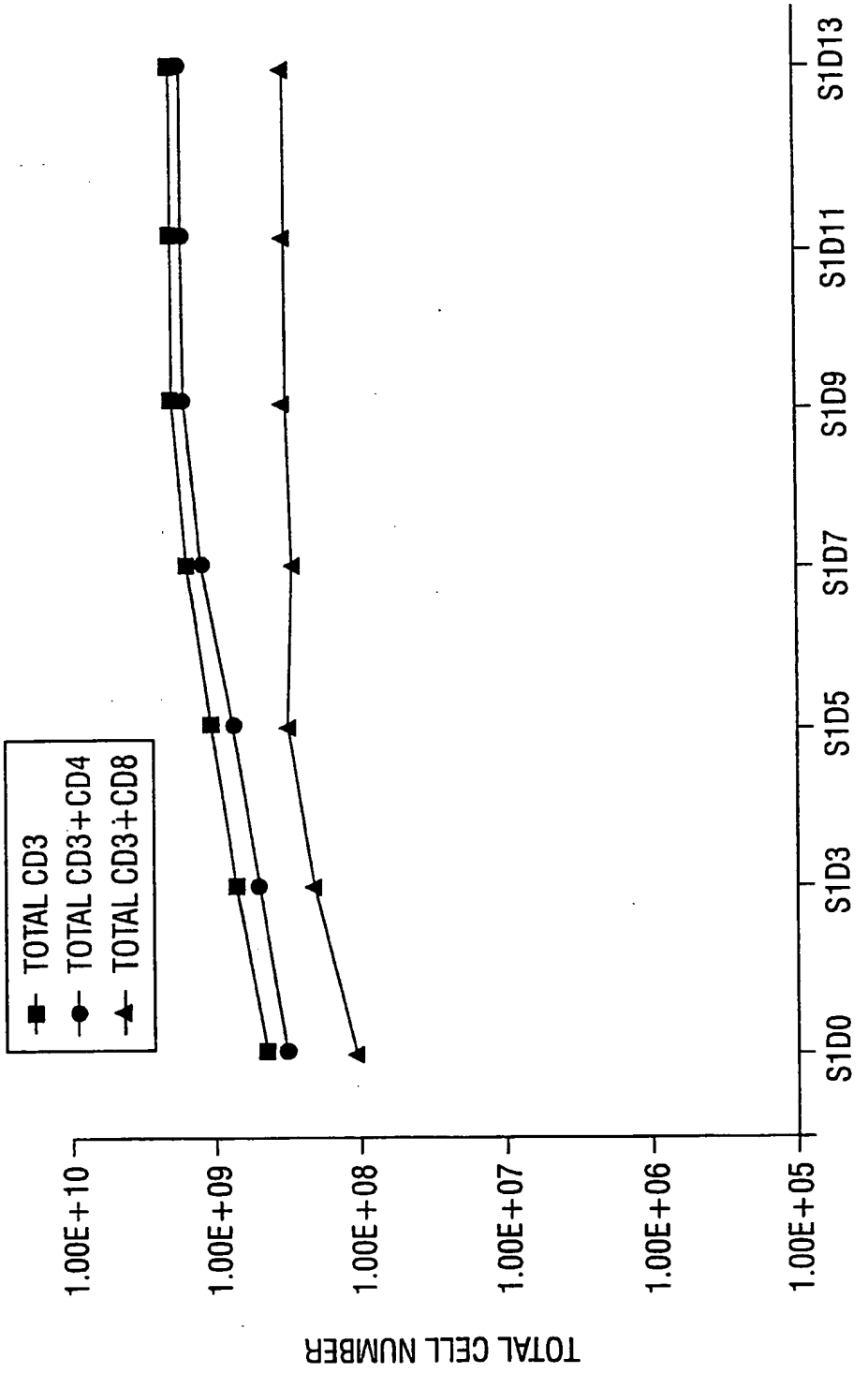

1E+09
1E+08
1E+07
1E+06
CELL NUMBER
TOTAL CELLS
CD3+CD4+CELLS
CD3+CD8+CELLS
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS OF STIMULATION

FIG. 1B


TOTAL CD3
TOTAL CD3+CD4
TOTAL CD3+CD8
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
TOTAL CELL NUMBER
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S1D3
S1D5
S1D7
S1D9
S1D11
S1D13
CULTURE DAY

FIG. 2

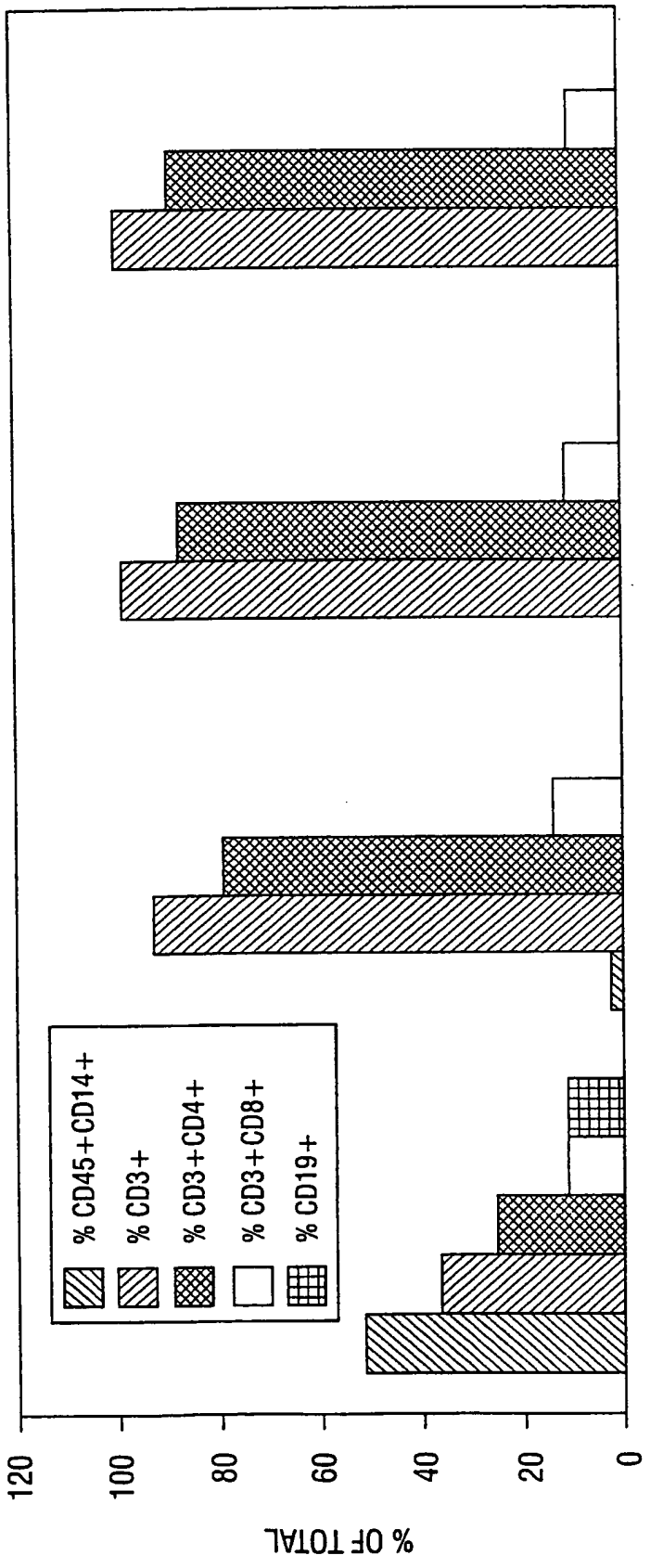
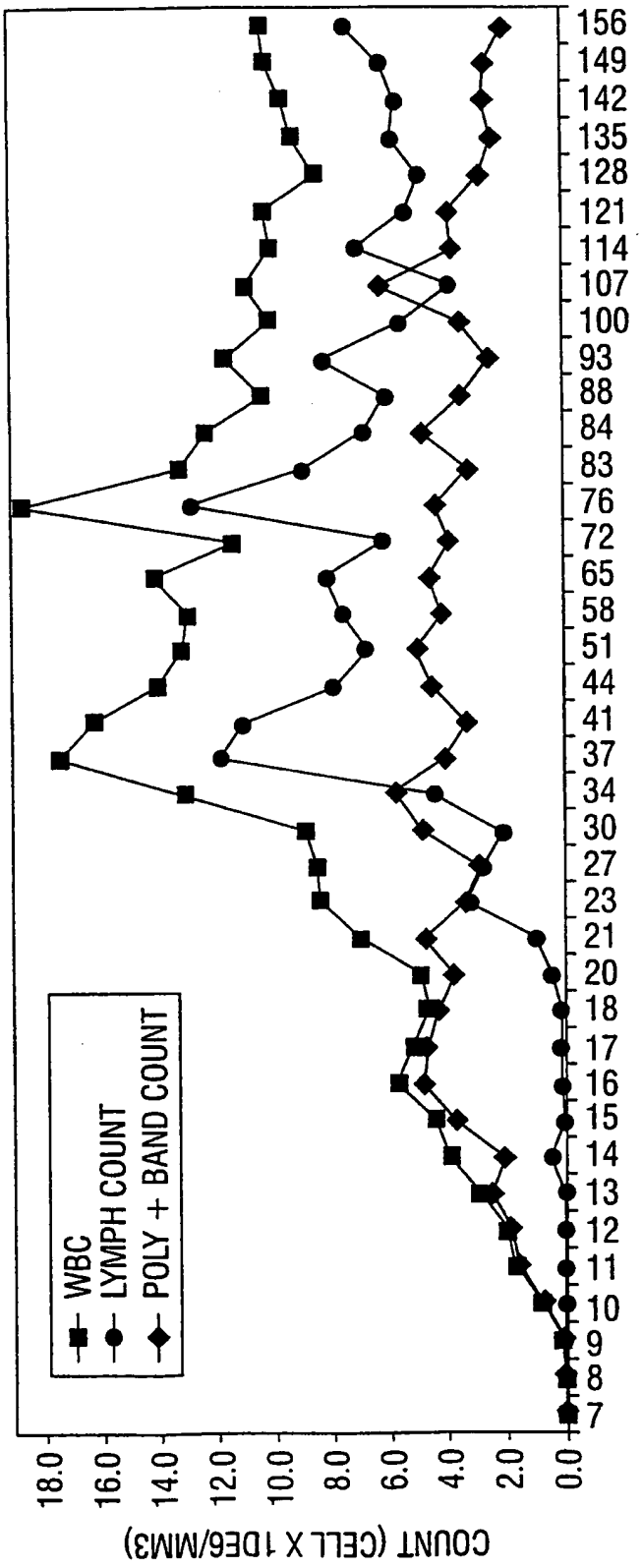

% CD45+CD14+
% CD3+
% CD3+CD4+
% CD3+CD8+
% CD19+
S1D0
S1D4
S1D9
S1D13
DAY OF STIMULATION
% OF TOTAL
120
100
80
60
40
20
0

FIG. 3


WBC
LYMPH COUNT
POLY + BAND COUNT
COUNT (CELL X 1DE6/MM3)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
156
149
142
135
128
121
114
107
100
93
88
84
83
76
72
65
58
51
44
41
37
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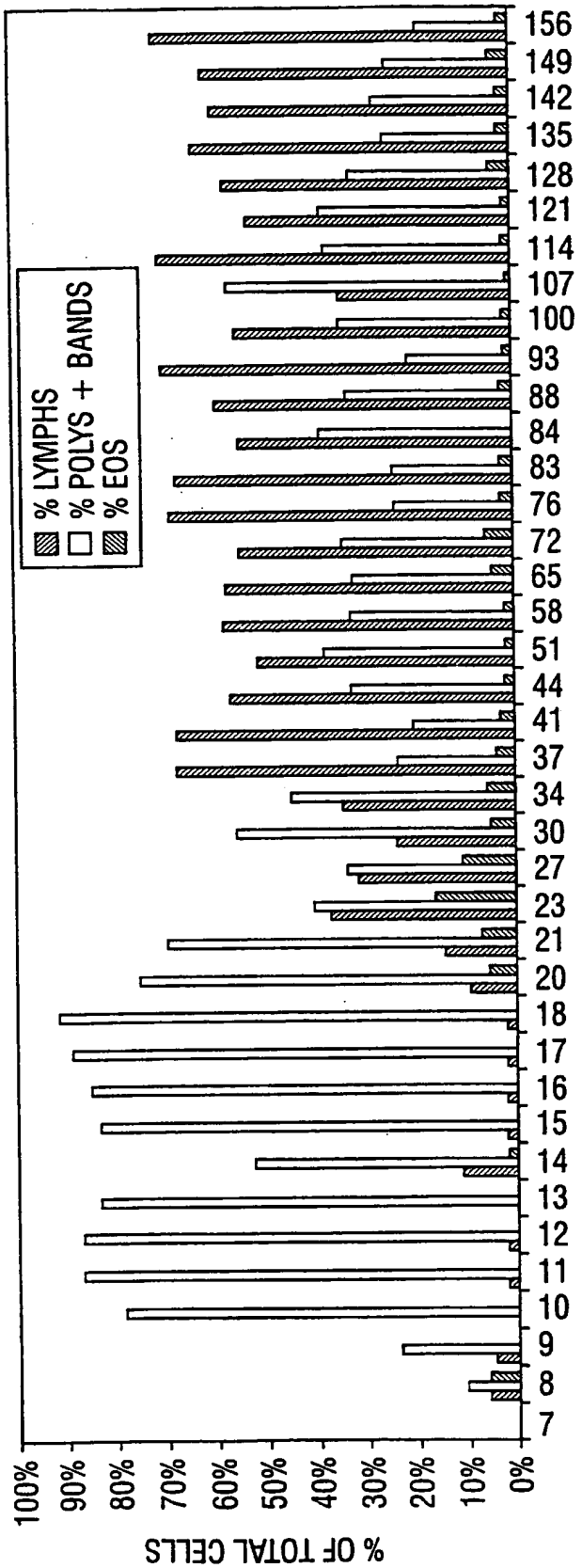
8
7

FIG. 4


% LYMPHS
% POLYS + BANDS
% EOS
156
149
142
135
128
121
114
107
100
93
88
84
83
76
72
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16
15
14
13
12
11
10
9
8
7
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% OF TOTAL CELLS

FIG. 5

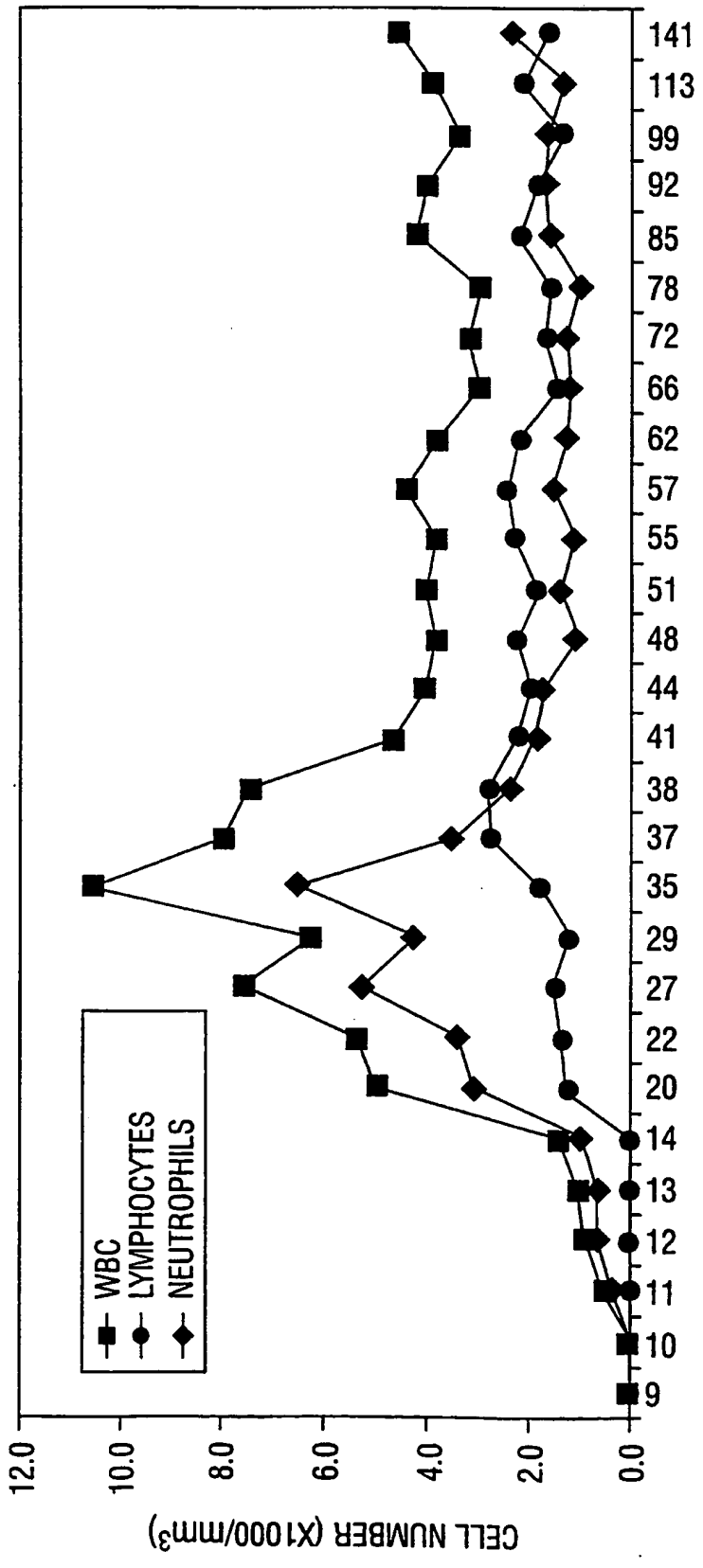
141
113
99
92
85
78
72
66
62
57
55
51
48
44
41
38
37
35
29
27
22
20
14
13
12
11
10
9
12.0
10.0
8.0
6.0
4.0
2.0
0.0
CELL NUMBER (X1000/mm3)
WBC
LYMPHOCYTES
NEUTROPHILS

FIG. 6

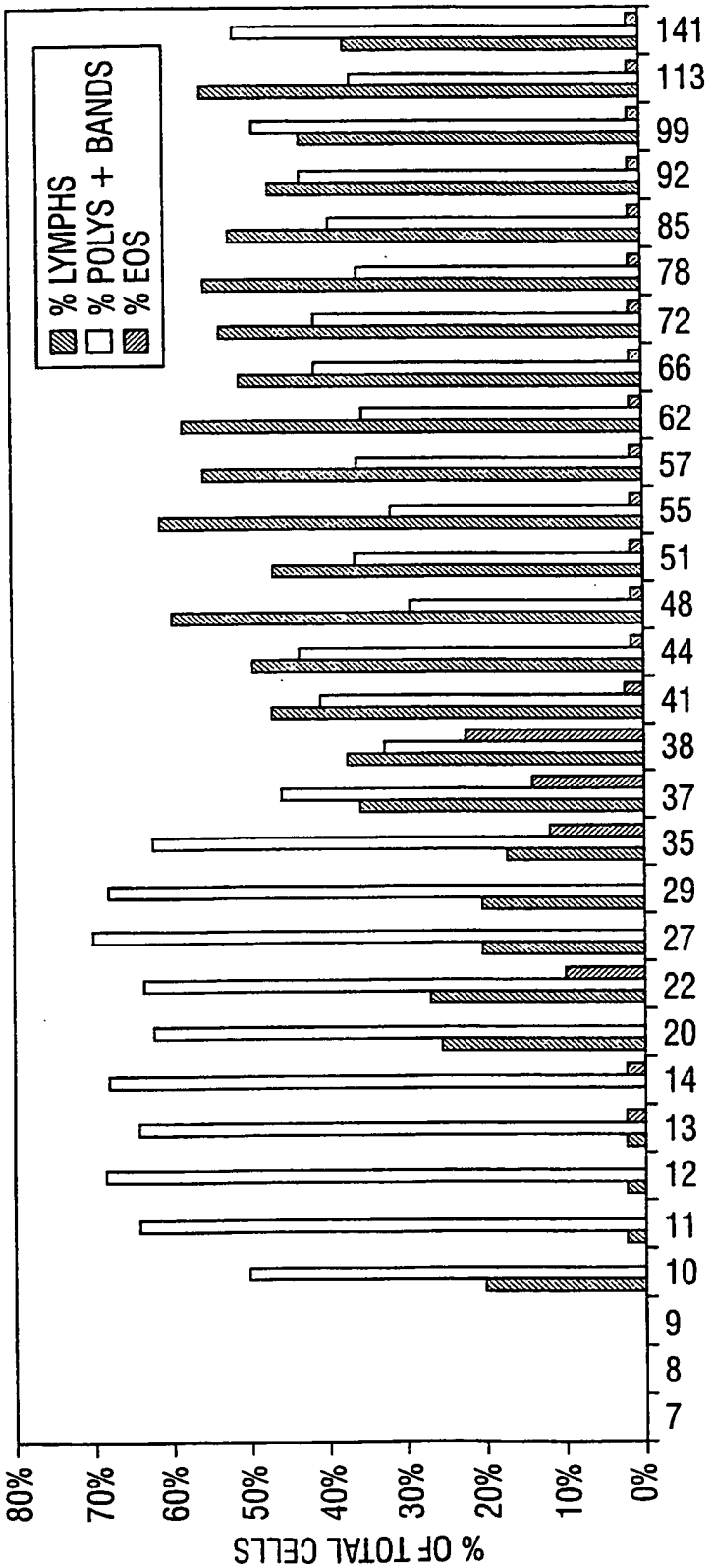
% LYMPHS
% POLYS + BANDS
% EOS
141
113
99
92
85
78
72
66
62
57
55
51
48
44
41
38
37
35
29
27
22
20
14
13
12
11
10
9
8
7
80%
70%
60%
50%
40%
30%
20%
10%
0%
% OF TOTAL CELLS

FIG. 7

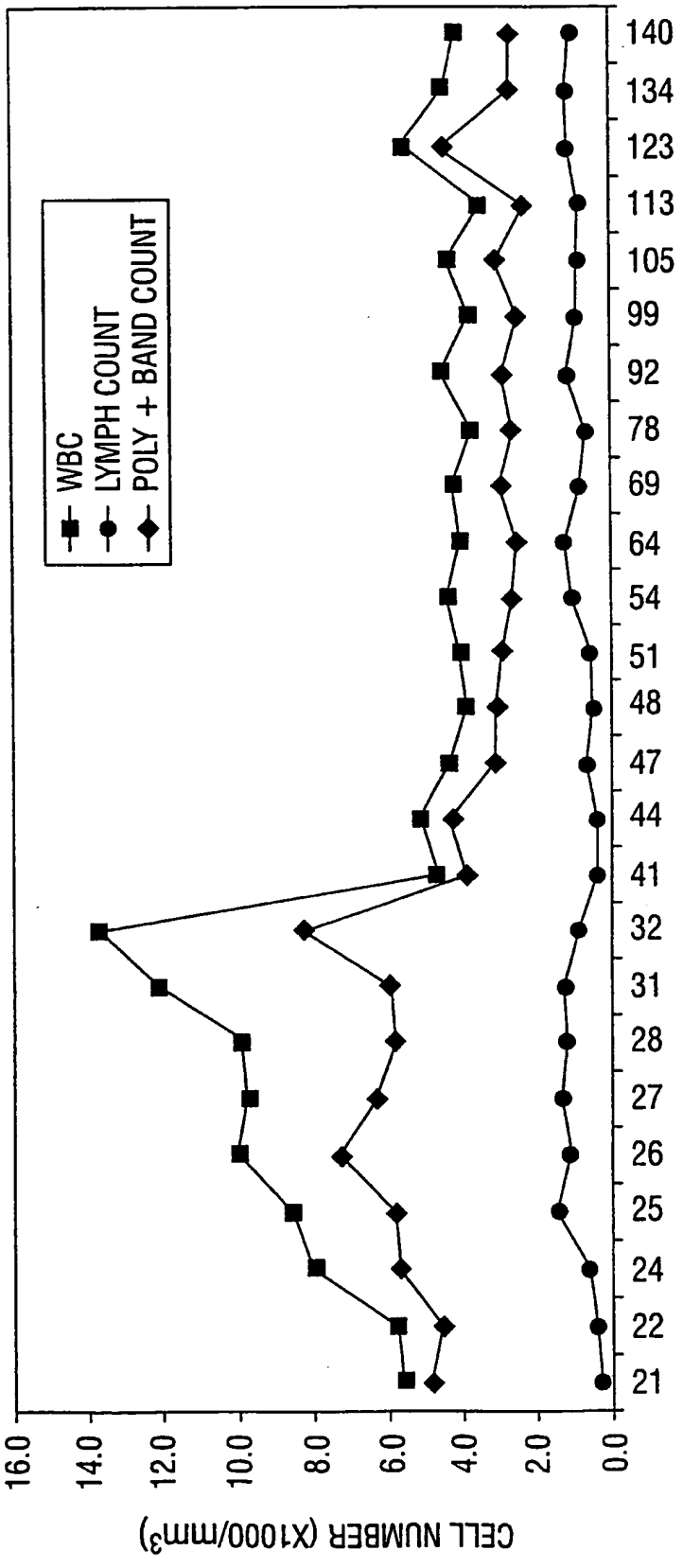
WBC
LYMPH COUNT
POLY + BAND COUNT
140
134
123
113
105
99
92
78
69
64
54
51
48
47
44
41
32
31
28
27
26
25
24
22
21
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
CELL NUMBER (X1000/mm3)

FIG. 8

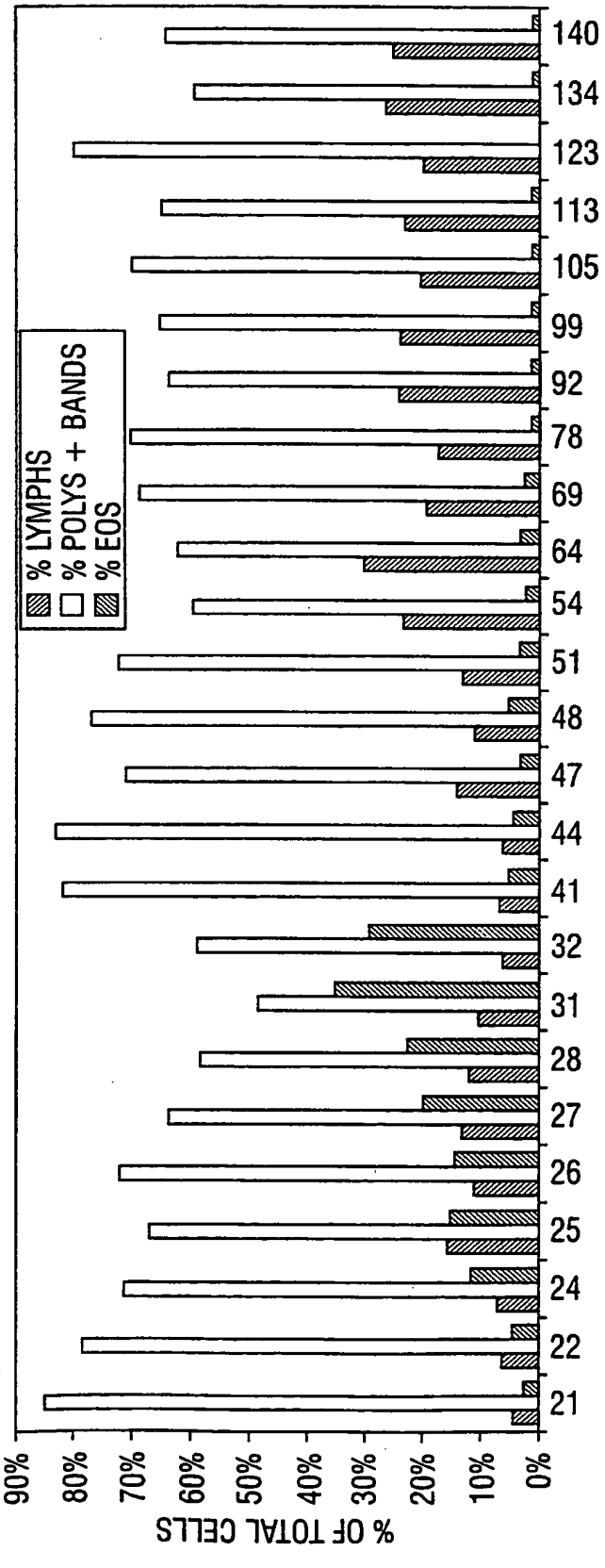
% LYMPHS
% POLYS + BANDS
% EOS
140
134
123
113
105
99
92
78
69
64
54
51
48
47
44
41
32
31
28
27
26
25
24
22
21
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% OF TOTAL CELLS

FIG. 9

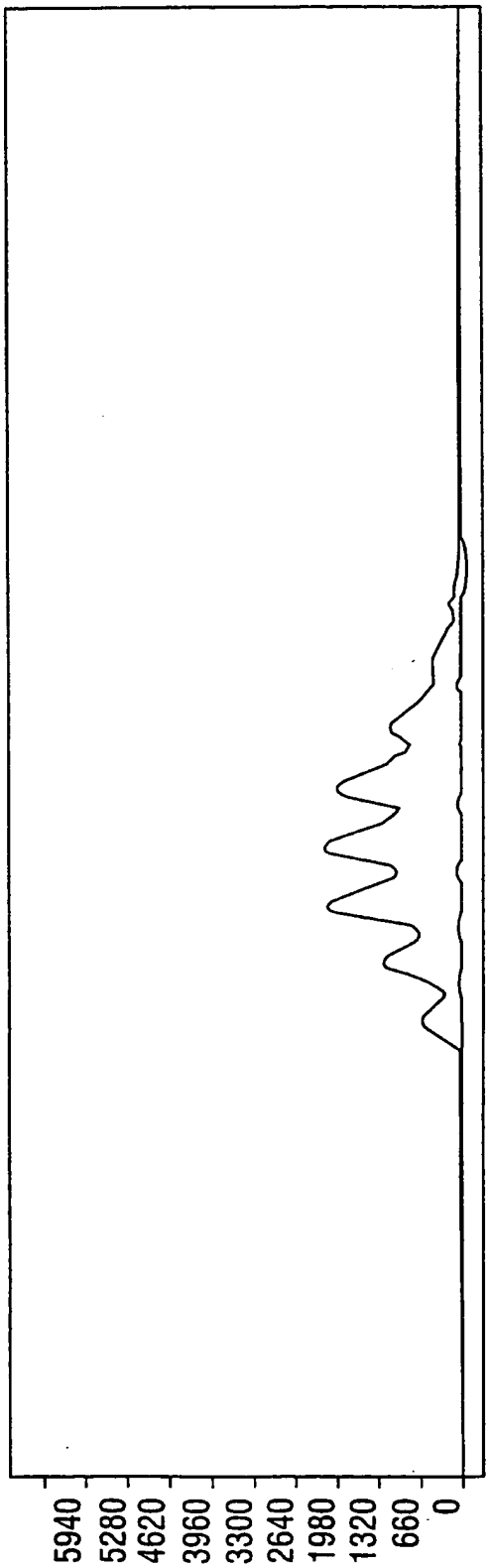

FIG. 10A

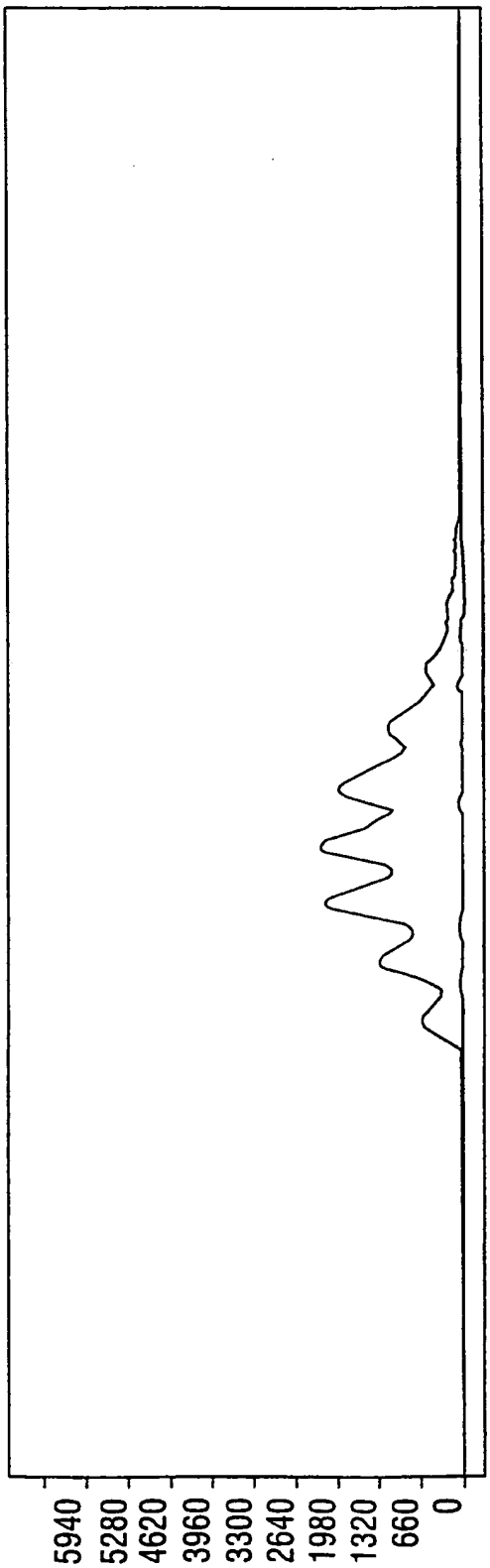

FIG. 10B

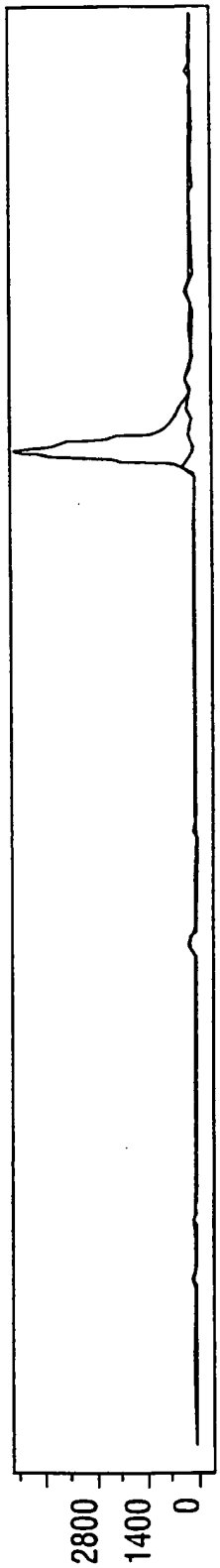

FIG. 10C

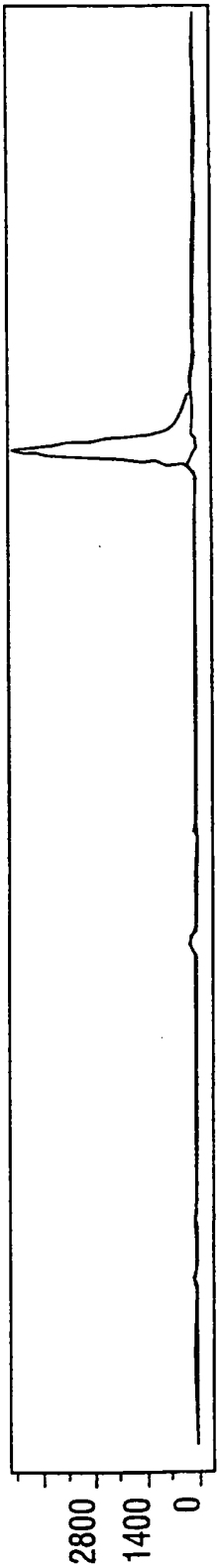

FIG. 10D

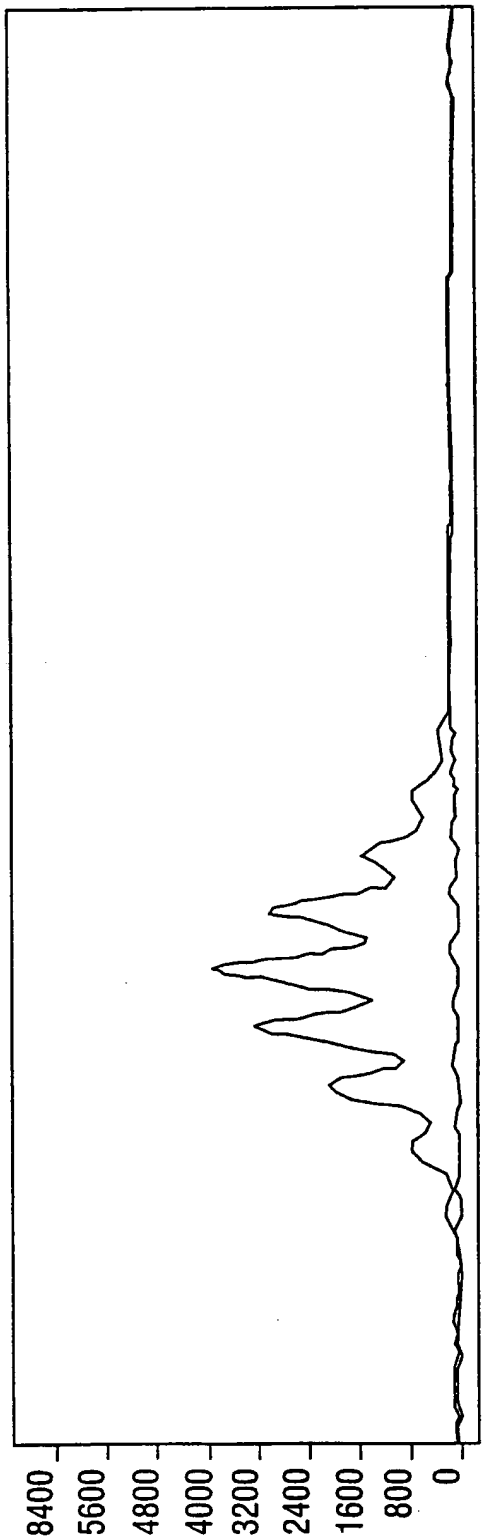

FIG. 11A

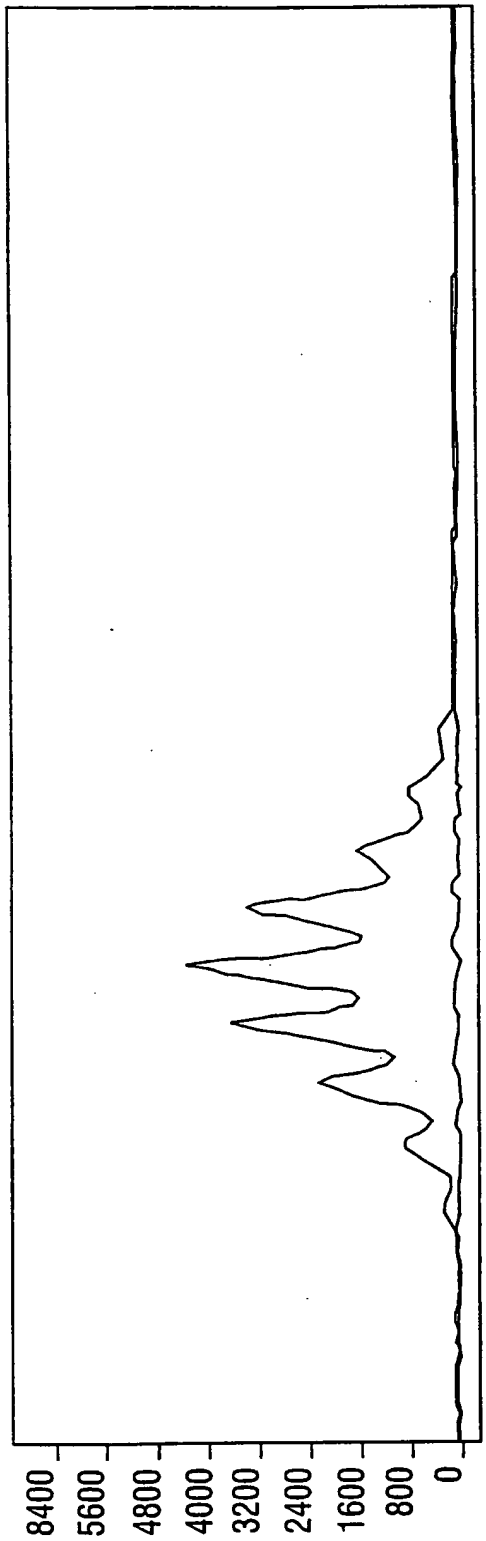

FIG. 11B

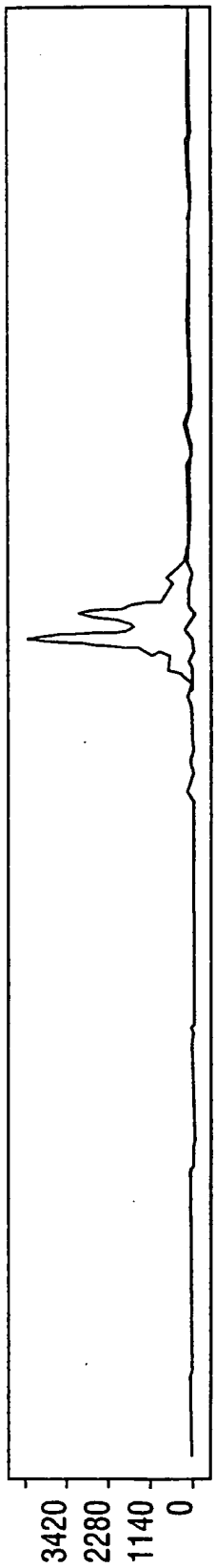

FIG. 11C

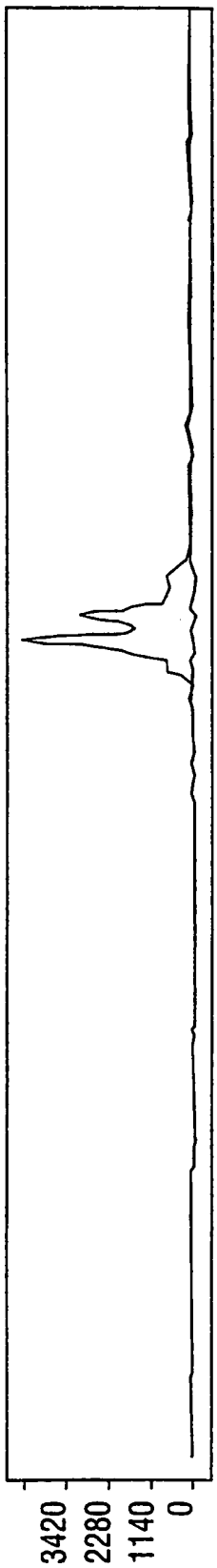

FIG. 11D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/23954

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/14 A61K45/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 267 615 A (SLOAN KETTERING INST CANCER) 18 May 1988 see whole doc. esp. claims, abstract and figures	1
Y	LEVINE B. L. ET AL.,: "Antiviral effect and ex vivo CD4+ T cell proliferation in HIV-positive patients as aresult of CD28 costimulation" SCIENCE, vol. 272, - 28 June 1996 pages 1939-1943, XP002095599 see whole doc., esp. fig.1 ; p.1940, col. 3; p.1942, col. 3	1-34

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1999	18/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/23954

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	MAZZONI A. ET AL.,: "CD3-CD28 costimualtion as a means to avoiding T cell preactivation in bisoecific monoclonal antibody-based treatment of ovarian carcinoma" CANCER RESEARCH, vol. 56, - 1 December 1996 pages 5443-5449, XP002095600 see whole doc. esp. discussion p.5448 ---	1-34
X	WO 97 05239 A (CELLTHERAPY INC ;GRUENBERG MICHEAL L (US)) 13 February 1997 see whole doc. esp. claims and p.2 l.15ff ---	1,2,8, 11-21, 31-33
X	WO 93 19767 A (UNIV MICHIGAN) 14 October 1993 see claims e.g. 65 and 81 ff, and p.12 l. 25 ff ---	1,2, 8-10, 12-14, 16,17, 21,22
A	WO 94 29436 A (REPLIGEN CORP ;UNIV MICHIGAN (US); US GOVERNMENT (US)) 22 December 1994 see whole doc. esp. claims and p.12, 125 ff. -----	1-34

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/23954

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-34 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/23954

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0267615 A	18-05-1988	AU 617295 B	28-11-1991
		AU 8117687 A	19-05-1988
		JP 1085930 A	30-03-1989
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WO 9319767 A	14-10-1993	AU 684461 B	18-12-1997
		AU 4101193 A	08-11-1993
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		EP 0637963 A	15-02-1995
		JP 7508711 T	28-09-1995
		US 5858358 A	12-01-1999
WO 9429436 A	22-12-1994	AU 7052194 A	03-01-1995
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		EP 0700430 A	13-03-1996
		JP 8511166 T	26-11-1996
		US 5858358 A	12-01-1999